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13. ABSTRACT *(Maximum 200 Words)*

c-myc is one of the most common oncogene aberrations in breast cancer, suggesting its important role in the genesis and/or progression of breast cancer. Therefore, it is critical to elucidate the precise molecular mechanisms of c-*myc* gene regulation. The goal of this proposal was to understand how potassium channel blocker, quinidine, regulates expression of c-*myc* oncogene in human breast cancer cells. We found that quinidine significantly suppressed c-*myc* promoter activity and a 168 bp region of human c-*myc* promoter, a quinidine response region (-100 to +68 with respect to the P1) is sufficient to confer responsiveness to quinidine. In addition, quinidine suppressed c-*myc* mRNA and protein levels in four human breast cancer but not normal breast epithelial cell lines. Suppression of Myc by quinidine paralleled by inhibition of growth and induction of a more differentiated phenotype in breast cancer cells. The preferential suppression of c-*myc* and induction of differentiation in tumor but not normal breast epithelial cells is very interesting and exciting finding. Quinidine is a potential lead compound for developing pharmacological agents to regulate Myc. In addition, the study of quinidine-regulated events is a promising approach to unravel differentiation control pathways that become disrupted in breast cancer.

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Table of Contents

Introduction

Previous studies demonstrated that quinidine causes G1/G0 cell cycle arrest and inhibition of proliferation in MCF-7 human breast cancer cell line (1). In addition, quinidine suppressed estradiol-stimulated c-*myc* mRNA levels in MCF-7 cells during 1-24 hours time course. The goal of this proposal was to elucidate the molecular mechanisms by which potassium channel blocking agent, quinidie, regulates the activity of c-*myc* gene. *c-myc* is one of the most common oncogene aberrations in breast cancer (2). *c-myc* functions include regulation of cell cycle, proliferation, differentiation, and apoptosis. The results of these studies demonstrated that quinidine causes rapid (within 1 hour) suppression of Myc protein and mRNA levels that precedes the quinidine-induced G1 cell cycle arrest point in MCF-7 cells. Additionally, the activity of *c-myc* promoter was suppressed by quinidine over the same range of concentrations that suppress levels of *c-myc* mRNA and protein, suggesting that changes in Myc protein and mRNA levels by quinidine may be attributed to its effect on *c-myc* promoter. A 168 bp region of *c-myc* promoter (-100 to +68 in respect to P1) was identified as a quinidine responsive region (QRR). Suppression of Myc by quinidine was consistent with inhibition of growth and induction of more differentiated phenotype in four different breast tumor cell lines. In contrast, quinidine had minimal effect on Myc levels or proliferation in MCF-10A normal mammary epithelial cell line. Furthermore, MCF-7 cells treated with *c-myc* antisense oligonucleotides exhibited cytoplasmic lipid droplets, similarly to the quinidine-treated cells, suggesting that suppression of Myc may play a causative role in the induction of more differentiated phenotype by quinidine in human breast cancer cells. Quinidine is a potential lead compound for developing pharmacological agents to regulate Myc. In addition, the study of quinidine-regulated events is a promising approach to unravel differentiation control pathways that become disrupted in breast cancer.

Body

The goal of this proposal was to understand the molecular mechanisms, by which potassium channel blocking agent, quinidine, regulates expression of c-*myc* gene in human breast cancer cells. The final approved Statement of Work (SOW) included the following specific aims:

The specific aim #1 consisted of three parts: 1A was to test the effects of quinidine on human c-*myc* gene promoter in a transient reporter gene assay; 1B was to construct a series of 5'-deletion mutants of the c-*myc* promoter to map a minimal region of the promoter that confers quinidine responsiveness (QRR-quinidine responsive region); 1C was to test the effects of quinidine on protein/DNA interactions within the QRR.
The specific aim #2 was to test the effects of quinidine on c-*myc* mRNA.
The specific aim #3 was to prepare and submit the manuscript(s).

Aim 1A: The ability of quinidine to inhibit c-*myc* gene promoter activity was tested using 2.8 kb human c-*myc* gene promoter region linked to the luciferase reporter gene (Del-1). MCF-7 human breast cancer cells were transiently transfected with the Del-1 plasmid. Cells were incubated in the presence of different concentration of quinidine for 24 hours following transfection, and luciferase activity was measured in the cell extracts. Luciferase activity in mock-transfected cells was less than 0.1% of the activity in control cells transfected with Del-1 (Figure 1A). Quinidine treatment caused a concentration-dependent decrease in the activity of the Del-1 *c-myc* promoter. Ninety μM quinidine decreased *c-myc* promoter activity by 60%.

Aim 1B: To better define the region of the *c-myc* promoter, that is responsive to quinidine (QRR-quinidine responsive region), a series of 5'-deletion mutants of Del-1 was tested (Figure 1B). The human *cyclin D1* gene promoter linked to the luciferase (CD1) and promoterless luciferase (Luc) plasmids were used as controls. Relative luciferase activity driven by the various plasmids was compared with Del-1. Quinidine suppressed the activity of all c-*myc* 5'deletion mutants by 60-70%, but had a minimal effect on luciferase expression driven by the *cyclin D1* promoter, suggesting that the effect of quinidine is promoter specific and not related to more general effects associated with cell cycle arrest in G1. Quinidine did not inhibit the enzymatic activity of the purified luciferase protein (data not shown). We conclude that quinidine suppresses the activity of the *c-myc* promoter, and that a 168 bp region of human c-*myc* promoter (QRR) from -100 to +68 with respect to the P1 promoter is sufficient to confer responsiveness to quinidine.

Aim 1C: The Quinidine Responsive Region (Figure 2) of c-*myc* promoter contains two important regulatory elements: transforming growth factor β1 (TGFβ1) control element (TCE) located between –83 and –63 in respect to P1 (3) and GC-rich region (from –60 to –37) that binds Sp1 and Sp3 transcription factors (4,5). The down-regulation of c-*myc* promoter activity through TCE has been reported to play the key role in TGF beta1 mediated growth inhibition in different cell lines. In order to test the role of TCE in the regulation of c-*myc* promoter by quinidine, PI carried out a series of experiments comparing effects of quinidine and TGF beta1 on cell proliferation and Myc protein levels in MCF-7, MDA-231, and MDA-468 human breast cancer cells and MCF10A normal human breast epithelial cells.

In MCF10A cells, TGF-β1 stimulates the formation of an inhibitory complex on the c-*myc* promoter and rapid down-regulation of *c-myc* mRNA, but this response is lost from MCF10A cells after transformation with c-Ha-*ras* and c-*erb*B2 oncogenes (6). TGF-β1 resistant MDA-468 breast tumor cells lack Smad4, a transcription factor and an essential TGFβ1 signaling

molecule (6). In MDA-231 cells, resistance to TGFβ is not fully understood, however, ectopic expression of TGF-β type III receptor suppressed tumorigenicity of MDA-231 cells (7). In contrast, MCF-7 cells have a partial response to TGFβ growth inhibition, and ectopic TGFβRIII restores autocrine TGF-β1 activity in MCF-7 cells (8). Despite these distinct defects in TGFβ1 signaling, quinidine inhibited proliferation in all breast tumor cell lines tested, implying that quinidine acts independently of TGF-β1 pathways (Figure 3). Furthermore, quinidine had no effect on the growth of MCF10A cells (Figure 4) in which the TGF-β1 pathway is intact. This result is important because it argues against the possibilities that quinidine acts either to restore a TGF-β1 response network, or to compensate for TGF-β1 defects. In Figure 4, the effects of quinidine and TGF-β1 on proliferation and Myc protein levels in a single experiment is summarized. We found (Figure 4) in this experiment that after 72 hours in quinidine MCF10A cell numbers decreased slightly (22%), TGF-β1 alone caused a 54% reduction in cell numbers, and the combination of quinidine plus TGFβ1 appeared to have an additive effect, reducing overall cell numbers by 72%. Similar reductions in the levels of Myc protein in MCF10A cells by quinidine (17%), TGF-β1 (54%), and the combination (Q+T, 67%) were observed (Figure 4B). The data support the hypothesis that quinidine and TGF-β1 act through independent mechanisms to reduce c-*myc* expression and cell proliferation.

By eliminating TCE as a mediator of quinidine's responses on c-*myc* promoter, the only described regulatory element remaining within the proposed 168 bp QRR is the GC-rich region. This region has been shown to be essential for P1 transcription (4,5) and can bind both Sp1 and Sp3 transcription factors. To assay the involvement of the GC-rich region in regulation of c-*myc* promoter by quinidine, PI next tested effects of quinidine on the protein levels of both transcription factors (Sp1 and Sp3) in MCF-7 cells. As shown in Figure 2, quinidine had no effects in either case. From these results the PI concluded that suppression of c-*myc* promoter activity in response to quinidine is not mediated by the changes in the levels of Sp1 or Sp3 transcription factors. In addition, our Electrophoretic Mobility Shift Assay (EMSA) showed no effect of quinidine on DNA-protein complex formation after incubating the nuclear extracts of control or quinidine-treated MCF-7 cells with radiolabeled DNA sequence corresponding to the Sp1/Sp3 binding site of the QRR (data not shown).

In summary, we demonstrated that quinidine significantly suppressed c-*myc* promoter activity and a 168 bp region of the human c-*myc* promoter (QRR) from -100 to +68 with respect to the P1 promoter is sufficient to confer responsiveness to quinidine. In addition, effects of quinidine on c-*myc* promoter were not mediated through TGFβ1 control element or GC-rich region of QRR. It is possible that suppression of c-*myc* promoter by quinidine is mediated by a novel regulatory element within QRR.

Aim #2 was to test the effects of quinidine on c-*myc* mRNA. Acute induction of *c-myc* mRNA transcription in MCF-7 cells exposed to estradiol is critical for progression through G1 phase of the cell cycle and the proliferative response to estradiol (9,10). At one hour, quinidine suppressed estradiol (E2)-induced *c-myc* mRNA levels in MCF-7 cells in a concentration dependent fashion. Maximum inhibition (60%) of c-*myc* mRNA was achieved with 90 μM quinidine (Figure 5A). Suppression of E2-induced c-*myc* mRNA levels paralleled by decrease in estradiol stimulated MCF-7 cell cycle progression into S phase (Figure 5B). Effects of quinidine on c-*myc* mRNA levels were not mediated by regulation of the c-*myc* mRNA stability (data not shown). To further corroborate that suppression of c-*myc* mRNA and protein levels in response to quinidine is mediated by its effect on c-*myc* promoter, the PI compared a dose-dependent

decrease in c-*myc* mRNA (Figure 6B), protein levels (Figure 6A) and promoter activity (Figure 1A). As shown in these figures, quinidine suppressed c-*myc* mRNA and protein levels in breast cancer cells over the same range of concentrations that suppressed levels of c-*myc* promoter.

In summary, we demonstrated that quinidine suppressed both E2-stimulated and basal c-*myc* mRNA levels in human breast cancer cells, which correlated with suppression of Myc protein levels and S phase progression. The effects of quinidine on c-*myc* mRNA and protein were mediated by suppression of c-*myc* promoter activity by quinidine, rather than its effect on c-*myc* mRNA stability.

Aim #3 was to prepare and submit the reports and manuscript(s). The results from this proposal enabled the PI to publish one first-author (International Journal of Cancer) and one second-author (Journal of Biological Chemistry) papers. In addition, the PI published and presented five abstracts at the national meetings (American Association for the Cancer Research, American Society for Cell Biology).

In addition to the data discussed above, the PI complemented this study with the following experiments due to the suggestions of the reviewers of the IJC manuscript:

1) Tested the effects of quinidine on Myc protein levels in other human breast cancer cell lines and normal human mammary epithelial cells, MCF10A. Our results demonstrated suppression of Myc protein levels in three other breast tumor cell lines (Figure 7, hatched bars): MCF-7*ras* (by 82 %), MDA-MB-231 (by 66%), MDA-MB-435 (by 59%). The degree of suppression of Myc protein levels by quinidine correlated well with the extent of inhibition of proliferation in each of these cell lines (Figure 7, solid bars). MCF10A is a rapidly proliferating but non-tumorigenic mammary epithelial cell line that expresses high Myc protein levels. In contrast to the tumor cell lines, quinidine had no effect on Myc protein expression or proliferation of MCF10A cells (Figure 7). These results demonstrate the ability of quinidine to selectively inhibit growth and Myc protein in tumorigenic breast cells but not in an immortalized, non-tumorigenic breast epithelial cell line.

2) Compared the effects of quinidine and c-*myc* antisense oligonucleotides on accumulation of cytoplasmic lipid droplets in MCF-7 human breast cancer cells. Lipid droplets are found in the cytoplasm of normal mammary epithelium (11), and induction of differentiation in human breast cancer cell lines by retinoic acid (12), vitamin D analog, 1-α- hydroxyvitamin D5 (13), and oncostatin M (14) is marked by the accumulation of cytoplasmic lipid droplets. Figure 8 summarizes a series of experiments in which differentiation in human mammary cells *in vitro* in response to either quinidine (90 μM) or c-*myc* antisense or sense oligonucleotides was evaluated by Oil Red O staining of cytoplasmic lipid droplets. Only normal human mammary epithelial cells (HMEC) showed cytoplasmic lipid droplets in control, untreated cultures (Figure 8G). HMEC exposed to quinidine (90 μM) for 72 h showed no increase in lipid droplet accumulation (Figure 8H), and no signs of cytotoxicity. The latter observation was consistent with our earlier report that indicated quinidine did not inhibit HMEC proliferation (15, Figure 6, see appended paper). MCF10A cells are immortalized human mammary epithelial cells that do not form tumors in animals (16). However, MCF10A cells proliferate more rapidly in culture (doubling time 37 h) than the early passage MCF-7 cells used in these experiments (doubling time 57 h). MCF10A cells provide one test for discriminating whether quinidine acts upon all cells with a rapid proliferation rate, or whether quinidine differentially affects tumorigenic mammary epithelial cells. Control MCF10A cells did not accumulate cytoplasmic lipid droplets,

and neither quinidine treatment or c-*myc* antisense oligonucleotides elicited this response (data not shown). In contrast, lipid droplet accumulation and cytoplasmic enlargement was evident in MCF-7 cells treated with 90 μM quinidine for 72 hours (Figure 8B) and with c-*myc* antisense for 96 hours (Figure 8E). Control (untreated) MCF-7 cells (Figure 8A) and MCF-7 cells exposed to c-*myc* sense oligonucleotides (Figure 8F) for 96 hours did not accumulate lipid droplets. We conclude that quinidine and c-*myc* antisense oligonucleotides promote a more differentiated phenotype in MCF-7 cells. Quinidine promoted lipid droplet accumulation in MDA-MB-231, MDA-MB-435, MCF-7*ras* and T47D cells (15, Figure 8, see appended paper) suggesting that this differentiation response to quinidine is common among human mammary tumor cell lines.

The preferential suppression of c-*myc* and induction of differentiation in tumor but not normal breast epithelial cells is very interesting and exciting finding. Quinidine is a potential lead compound for developing pharmacological agents to regulate Myc. In addition, the study of quinidine-regulated events is a promising approach to unravel differentiation control pathways that become disrupted in breast cancer.

The following outline summarizes an up-to-date progress for the each specific aim:

Aim 1A	Months 1-2	Completed
Aim 1B	Months 3-24	Completed
Aim 1C	Months 24-26	Completed
Aim 2	Month 26	Completed
Aim 3	Months 24-30	Completed

List of personnel receiving pay from the research effort:

- Zaroui K. Melkoumian, Ph.D. (PI)

Key research accomplishments:

- Completion of the specific aims # 1,2, and 3 of the USAMRMC Breast Cancer Research Program Predoctoral training grant.

Reportable outcomes:

Research Papers

Melkoumian ZK, Martirosyan AR, Strobl JS. Myc protein is differentially sensitive to quinidine in tumor versus immortalized breast epithelial cell lines. *International Journal of Cancer* (in press).

Zhou Q, **Melkoumian Z**, Lucktong A, Moniwa M, Davie J, and Strobl J. Rapid Induction of histone acetylation and cellular differentiation in human breast tumor cell lines following degradation of histone deacetylase-1. *J. Biol. Chem.*, 275: 35256-35263, 2000.

Abstracts:

Melkoumian ZK, McCracken MA, Strobl JS. Suppression of c-Myc protein and induction of cellular differentiation in human breast cancer cells but not in the normal human breast epithelial cells by quinidine. *American Society for Cell Biology*, # 2023, 2001.

Melkoumian ZK, Strobl JS.: Suppression of c-myc mRNA levels and G0/G1 arrest of MCF-7 and MCF-7ras human breast cancer cells in response to quinidine. *Proc. American Assoc. Cancer Research.*188, 1999.

Zhou Q, **Melkoumian ZK**, Strobl JS. Quinidine activates $p21^{WAF\text{-}1/CIP\text{-}1}$ expression and phosphorylation of pRb prior to onset of apoptosis in MCF-7 human breast cancer cells. *Proc. American Assoc. Cancer Research.* 1503, 1999.

Melkoumian ZK, Strobl JS.: Quinidine suppresses c-myc promoter activity and induces differentiation of MCF-7 human beast cancer cells. *American Society for Cell Biology.* 2483, 1999.

Johnson DN, **Melkoumian ZK**, Lucktong A, and Strobl JS: Differentiation of human breast tumor cell lines by quinolines. *Molecular Targets and Cancer Therapeutics, AACR-NCI-EORTC, Washington, DC.* 437, 1999.

Degree and employment obtained:

December, 2001	Ph.D. in Pharmacology and Toxicology, West Virginia University
May, 2002- present	Postdoctoral Associate, Dept. Molecular Medicine, Cornell University Research area: Study the role of Focal Adhesion Kinase in breast tumorigenesis and metastatic progression

Conclusions:

C-myc is one of the most common oncogene aberrations in breast cancer suggesting its importance in the genesis and/or progression of breast cancer (1). The goal of this proposal was to understand how quinidine regulates expression of c-*myc* oncogene in human breast cancer cells.

Our results demonstrated that quinidine significantly suppressed c-*myc* gene promoter activity and a 168 bp region of human c-*myc* promoter, a quinidine response region (QRR), from -100 to +68 with respect to the P1 promoter is sufficient to confer responsiveness to quinidine. In addition, effects of quinidine on c-*myc* promoter were not mediated through TGFβ1 control element or GC-rich located within the QRR. It is possible that suppression of c-*myc* promoter by quinidine is mediated by the unknown regulatory element within the QRR.

In addition, quinidine suppressed both E2-stimulated and basal c-*myc* mRNA levels in human breast cancer cells, which correlated with the decrease in Myc protein levels and S phase progression. Suppression of c-*myc* mRNA and proteins levels was mediated by the effects of quinidine on c-*myc* promoter activity rather than affecting the stability of c-*myc* mRNA. Furthermore, quinidine decreased Myc protein levels in three other human breast cancer cell lines, but not in MCF10A normal mammary epithelial cells. Suppression of c-*myc* in breast tumor cells correlated with inhibition of growth and induction of a more differentiated phenotype.

One of the major problems of the current chemotherapeutic drugs for breast cancer treatment is their lack of selectivity for tumor tissue and associated with that toxicity in normal tissues. Therefore, the fact that quinidine selectively inhibits c-*myc* gene expression, cell growth and induces cellular differentiation in several different human breast cancer cell lines but not in normal breast epithelial cells is very interesting and exciting finding. Quinidine is a potential lead compound for developing pharmacological agents to regulate Myc. In addition, the study of quinidine-regulated events is a promising approach to unravel differentiation control pathways that become disrupted in breast cancer.

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Appendices

Figure Legends:

Figure 1. Suppression of *c-myc* promoter activity by quinidine.

A. MCF-7 cells were transfected with 5 μg/dish of Del-1 (-2265 myc) *c-myc*-luciferase reporter plasmid or with the transfection mix alone (DOTAP). At the end of transfection cells were incubated for 24 hours in DMEM/5%FBS + the indicated concentrations of quinidine before preparing cell extracts for luciferase assay. Data shown are the mean luciferase activity +/- S.D. of n =3 experiments (90 and 120μM quinidine) and n=2 experiments (30 and 50μM quinidine). The data are expressed as a percent of luciferase activity in control cells (100%). Luciferase activity in mock-transfected cells (DOTAP) was < 0.01% that of control cells.

* Significantly different from control (0 quinidine) cells ($p<0.05$)

B. Structures of the reporter plasmids. *c-myc* and *cyclin D1* promoter regions are solid; the luciferase coding region is indicated in white. P1 and P2 are the sites of the transcription initiation from the respective *c-myc* promoters. The nucleotide locations of 5' and 3'-ends of the myc-luc constructs are given with respect to the P1.

The effect of quinidine on *c-myc* promoter activity in 5'-deletion mutantsis shown to the right of each promoter structure. Cells were transfected with 5 μg/dish of *c-myc*-luc (Del-1, Del-2, Del-4, Frag-E) or *cyclinD1*-luc (CD1) reporter plasmids. At the end of transfection cells were incubated for 24 hours in DMEM/5%FBS +/- 90 uM quinidine before preparing cell extracts for luciferase assay. Luciferase activity driven by Del-1 in the absence of quinidine was set equal to one, and the activity of all the other promoters ± 90 μM quinidine was compared to 1. The percent change in luciferase activity by quinidine for each promoter is indicated. The range of luciferase cpm in MCF-7 cell extracts in these experiments were: Del-1 (200-500) and Del-4 (2800-6000).

* Significantly different from control values ($p<0.05$). Luciferase activity in cells transfected with promoterless construct was less than 2 % of the activity in control cells transfected with Del-1. Data are the mean +/- S.D. of at least n=3 experiments.

Figure 2. The structure of the QRR.

Figure 3. Effects of quinidine and TGF-β1 on cell growth.

Confluent cells were sub-cultivated in DMEM/5% FBS at the density of $1x10^5$ (MDA-231, MDA-468, MCF-10A) or $2x10^5$ (MCF-7) per 35 mm^2 tissue culture dish in the presence of: C-vehicle (4mM HCl, 1mg/ml BSA - 0.5 μl/ml of medium), Q- 90 μM quinidine, T-5ng/ml TGFβ1, T+Q-90 μM quinidine + 5ng/ml TGFβ1. Cells were harvested 72 or 96 (for MCF-7) hours later and counted using a hemocytometer. The bar graphs show cell numbers as mean +/- S.D. of n=2 experiments. Data are the mean +/- S.D. of n=1 experiments performed in triplicates.

Figure 4. Quinidine and TGFβ1 have distinct effects on proliferation and Myc protein levels in MCF10A cells.

Confluent MCF10A cells were sub-cultivated in MEGM +/- vehicle (4mM HCl, 1mg/ml BSA - 0.5 μl/ml of medium), 90 μM quinidine, 5ng/ml TGFβ1, 90 μm quinidine + 5ng/ml TGFβ1. Cells were harvested 24 (Western blot assay) and 72 (cell count assay) hours later. Myc protein levels were determined by densitometry and normalized to the β-catenin signals. The bar graph shows cell number

(solid bars) and Myc protein levels (hatched bars) in control and treated cells. The data in the table represent mean +/- STDEV of n=2 experiments.

Figure 5. Quinidine suppresses acute induction of *c-myc* mRNA and S phase progression by estradiol.

A. Confluent MCF-7 cells were sub-cultivated in PRF-DMEM/2% stripped serum, and estrogen-depleted for 40 hours. Induction of c-myc mRNA by 2 nM estradiol +/- indicated concentrations of quinidine was measured after 1 hour by isolating total cellular RNA and Northern blotting. c-*myc* mRNA signals were normalized to the 28S ribosomal signal in the ethidium bromide stained gel. Data shown on the bar graph are the mean +/- S.D. of n = 3, except for 30 and 50 μM quinidine that are from a single experiment. Values for E_2 and Q+E_2 treated groups are reported relative to control group (control=1).

B. MCF-7 cells were treated with C-0.01% Ethanol (vehicle), Q- 90 μM quinidine, E_2- 2nM estradiol, Q+E_2- 90 μM quinidine + 2nM E_2. Cell cycle phase distribution was analyzed by flow cytometry 30 hours later. Data are the mean +/- S.D. of n = 4 experiments.

* Significantly different from control cells ($p<0.05$).

Significantly different from E_2 cells ($p<0.05$).

Figure 6. Quinidine suppresses basal *c-myc* mRNA and protein expression in MCF-7 and MCF-7ras cells.

Confluent MCF-7 (solid bars) and MCF-7ras (hatched bars) cells were sub-cultivated into DMEM/5% FBS +/- indicated concentrations of quinidine. Cells were harvested 24 hours later and Myc protein (A) or mRNA (B) levels were analyzed by Western or Northern blots, respectively. Myc protein and mRNA signals were normalized to the β-catenin and 18S ribosomal RNA signals, respectively. Data shown on the bar graphs are the mean +/- S.D. of n= 4 (MCF-7) and n=3 (MCF-7ras) experiments for the panel A or n=1 for the panel B.

* Significantly different from control values ($p<0.05$).

Figure 7. Quinidine suppresses Myc induction and cell growth in human breast cancer cells in vitro, but not that of non-tumorigenic mammary epithelial cells.

For cell count assay (solid bars) confluent cells were sub-cultivated at the density of $1x10^5$/35 mm dish in DMEM/5%FBS +/- 90 μM quinidine or MEGM (for MCF-10A) +/- 90 μM quinidine and counted 96 hours later using a hemocytometer.

For Western blot assay (hatched bars) total cellular proteins from control or 90 μM quinidine treated cells were extracted one (MCF-7ras, MCF-7, MCF10A) or two (MDA-231, MDA-435) hours after sub-cultivation of confluent cells into DMEM/5% FBS. Myc protein signals were quantified by densitometry and normalized to the β-catenin signals.

Data are the mean +/- S.D. of n=3 experiments.

* Significantly different from control values ($p<0.05$).

Figure 8. Cellular differentiation of MCF-7 cells in response to c-myc antisense oligonucleotides and quinidine.

Oil Red O staining of MCF-7 cells treated with nothing (A and D), 90 uM quinidine for 72 (B) or 120 (C) hours, *c-myc* antisense (E) or sense (F) oligonucleotides for 96 hours. Oil Red O staining of normal human mammary epithelial cells, HMEC, control (G) or treated with 90 uM quinidine (H) for 72 hours. Cells were counterstained with hematoxylin to visualize the cell nuclei (blue color).

Melkoumian, Z.K.

Figure 1A

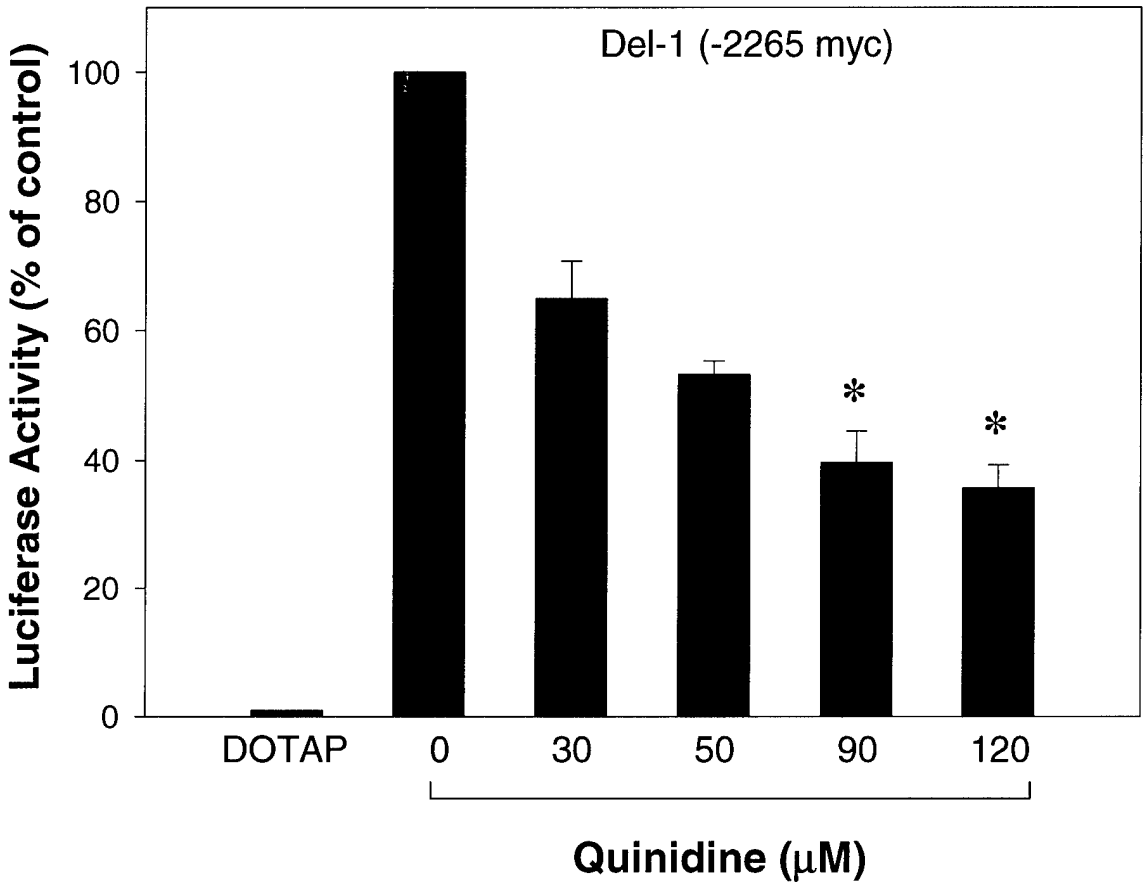


Figure 1B

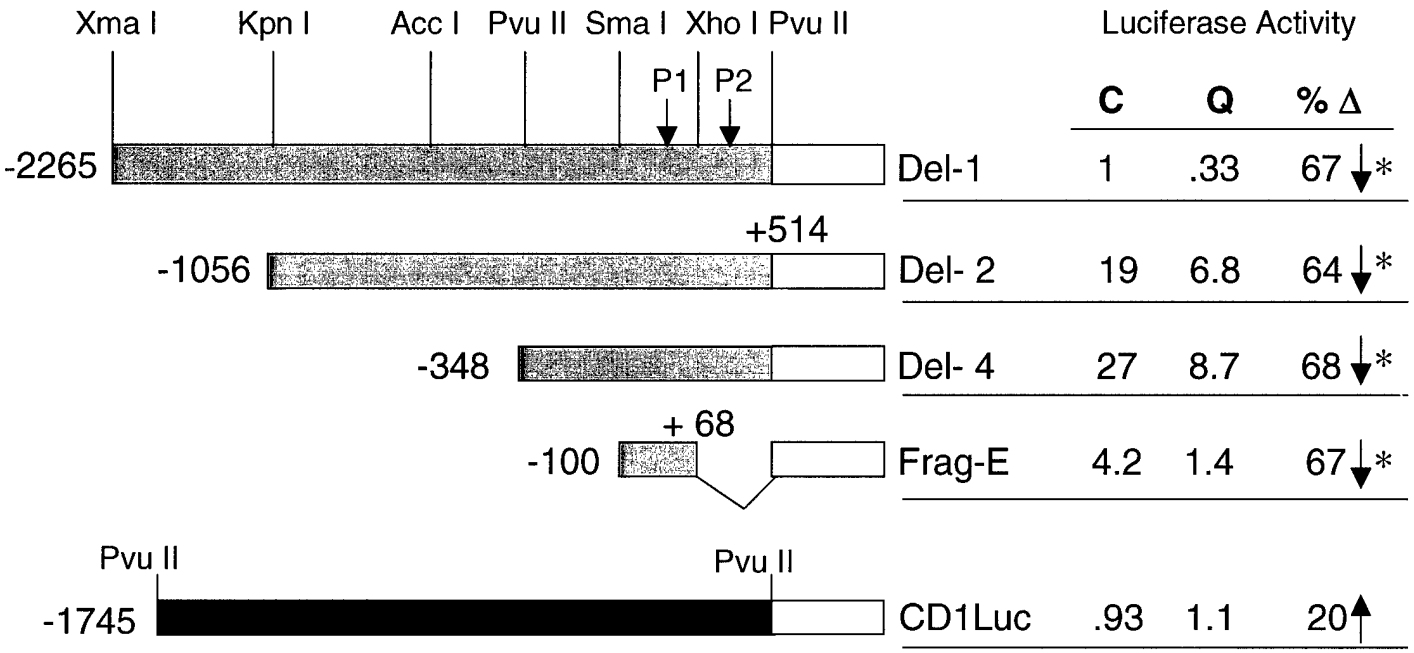


Figure 2A. The Structure of the 168 bp Quinidine Responsive Region (QRR) of the human c-myc gene promoter

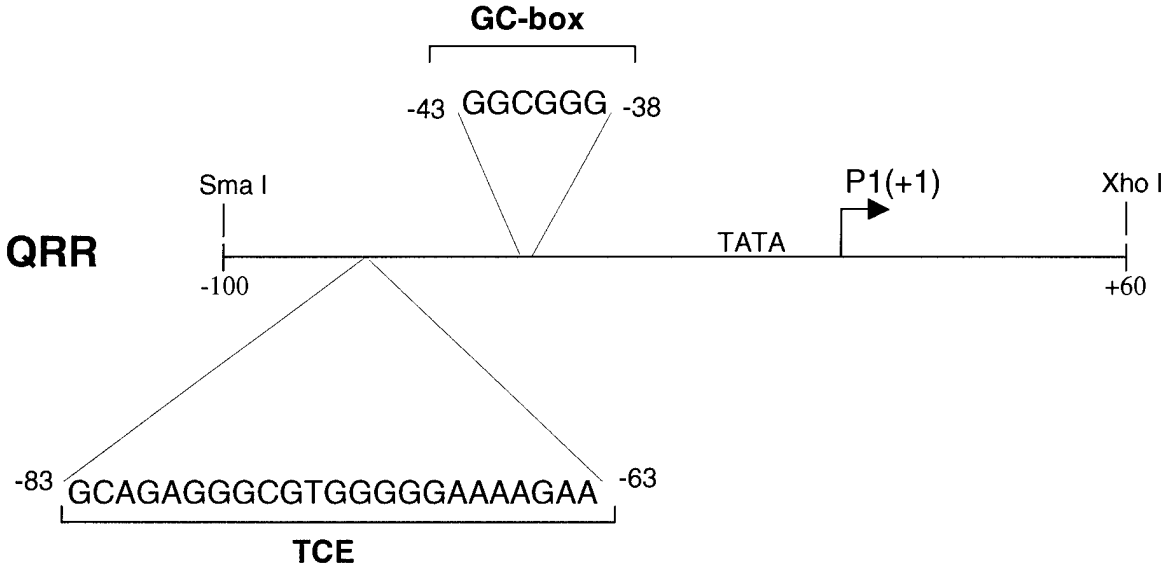
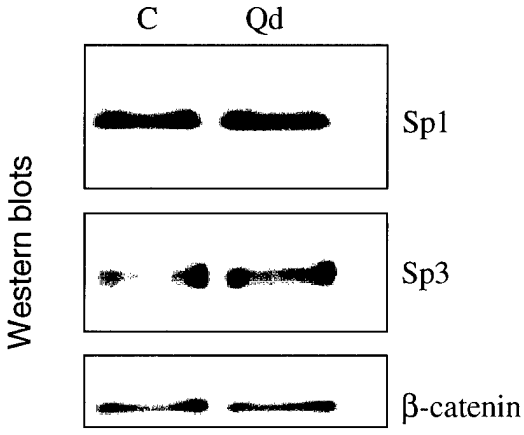


Figure 2B. Effects of 90 uM quinidine on Sp1 and Sp3 proteins levels in MCF-7 cells



The total cellular proteins from MCF-7 cells treated with +/- 90 uM quinidine were extracted 1.5 hrs after the plating. Western blot assays were performed to test the effects of quinidine on Sp1 and Sp3 proteins levels. The β-catenin signals were used as a loading control.

Figure 3

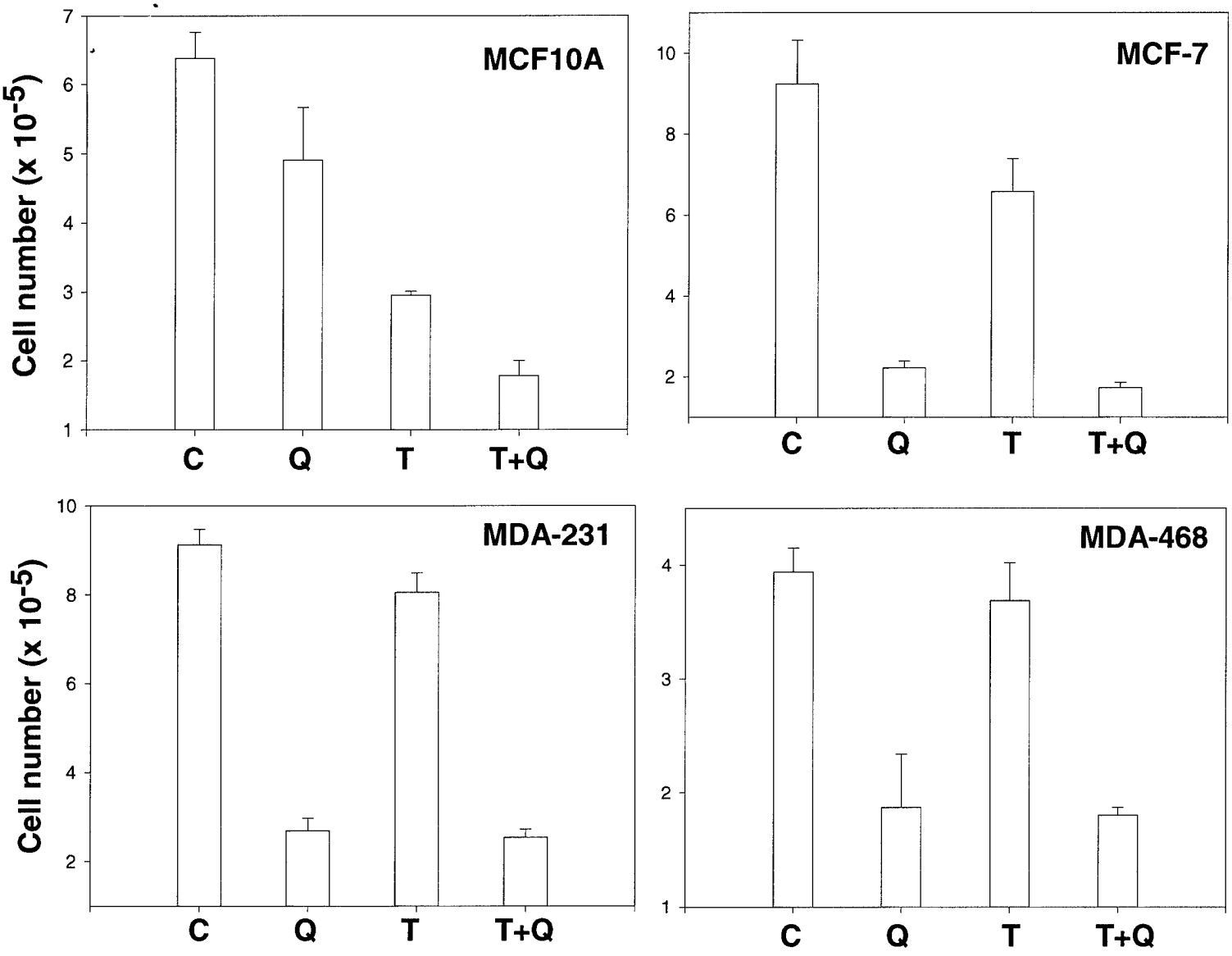
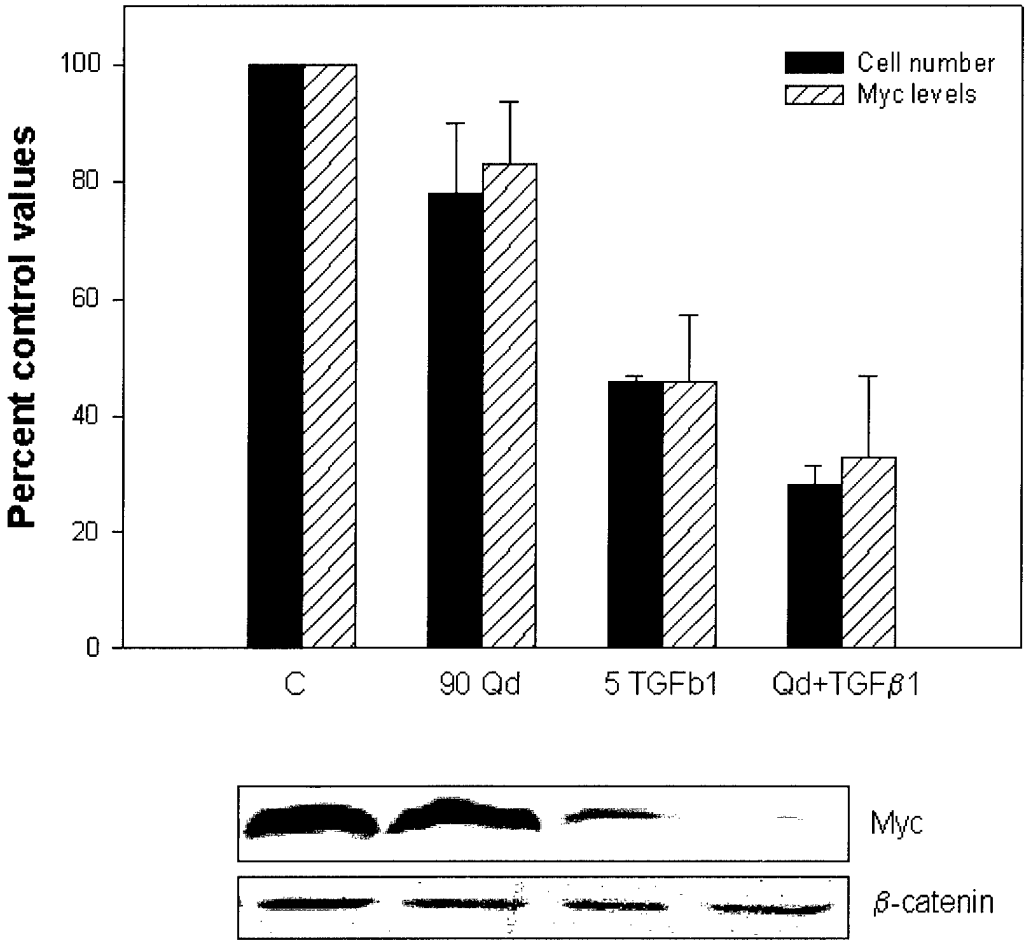


Figure 4



	Contorl	90 uM quinidne	5ng/ml TGFb1	90 Qd + 5TGFb1
Cell count data (% of control)	100	78 (12)	46 (0.9)	28 (3.4)
Myc protein levels (% of control)	100	83 (10.6)	46 (11.2)	33 (14.5)

Figure 5A

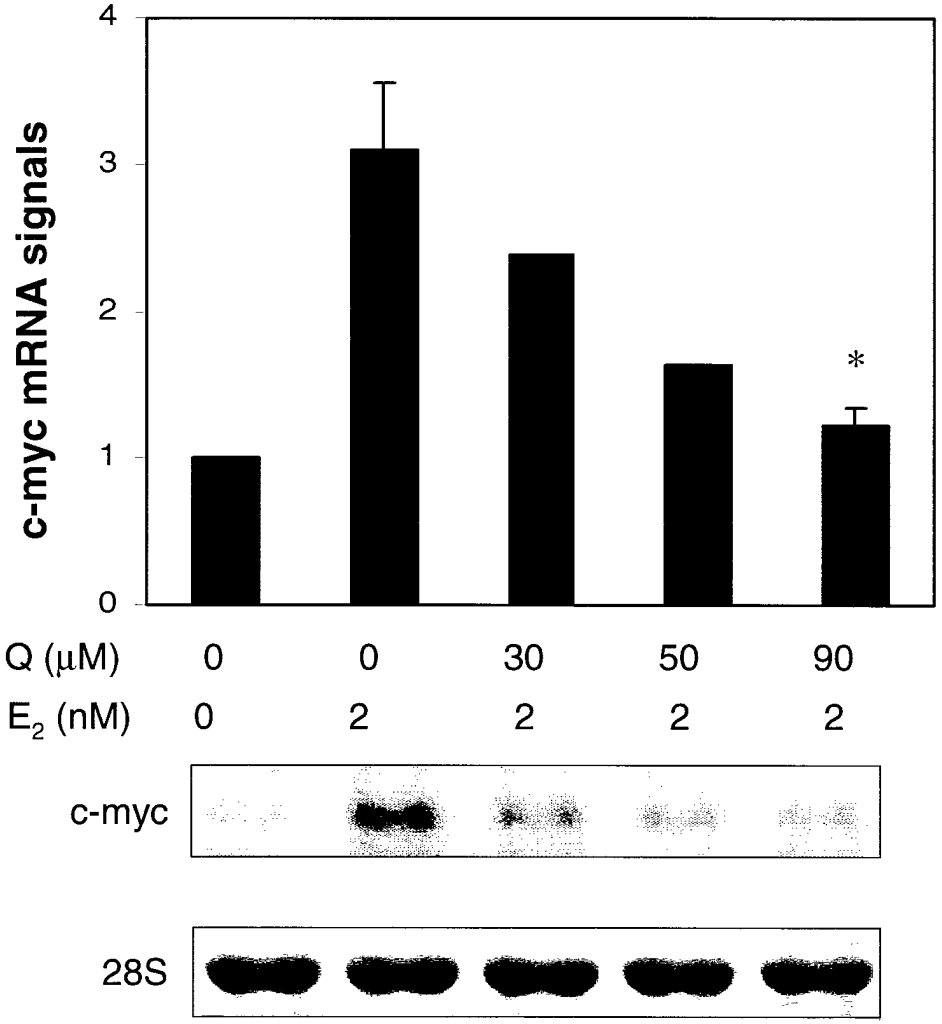
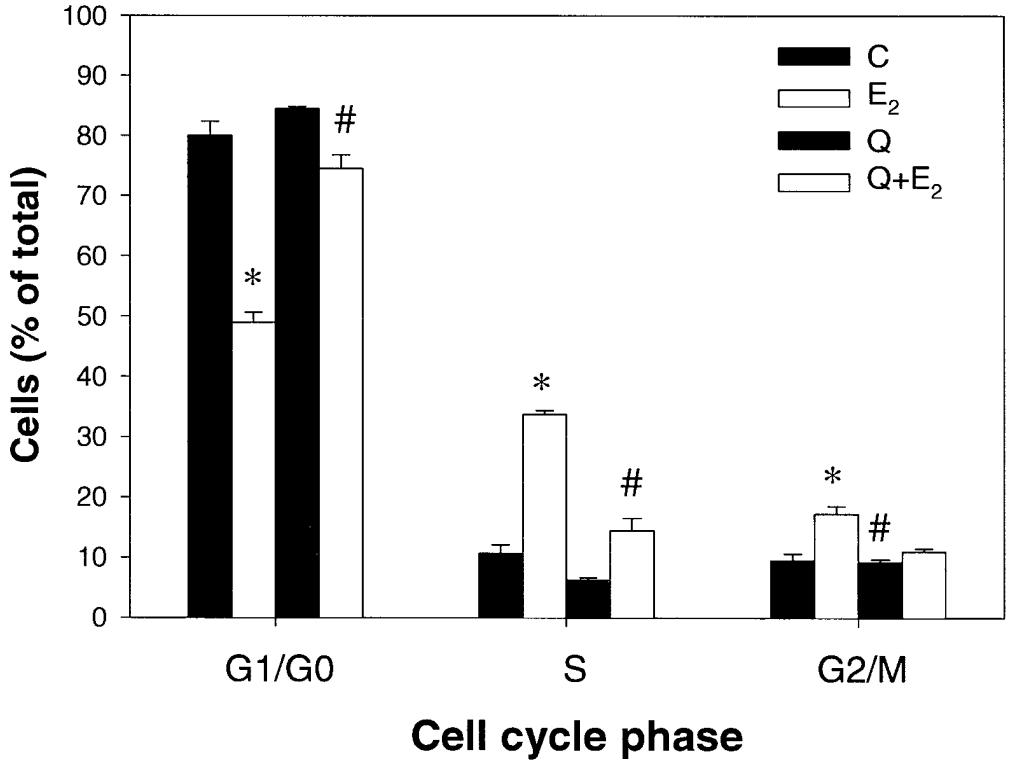
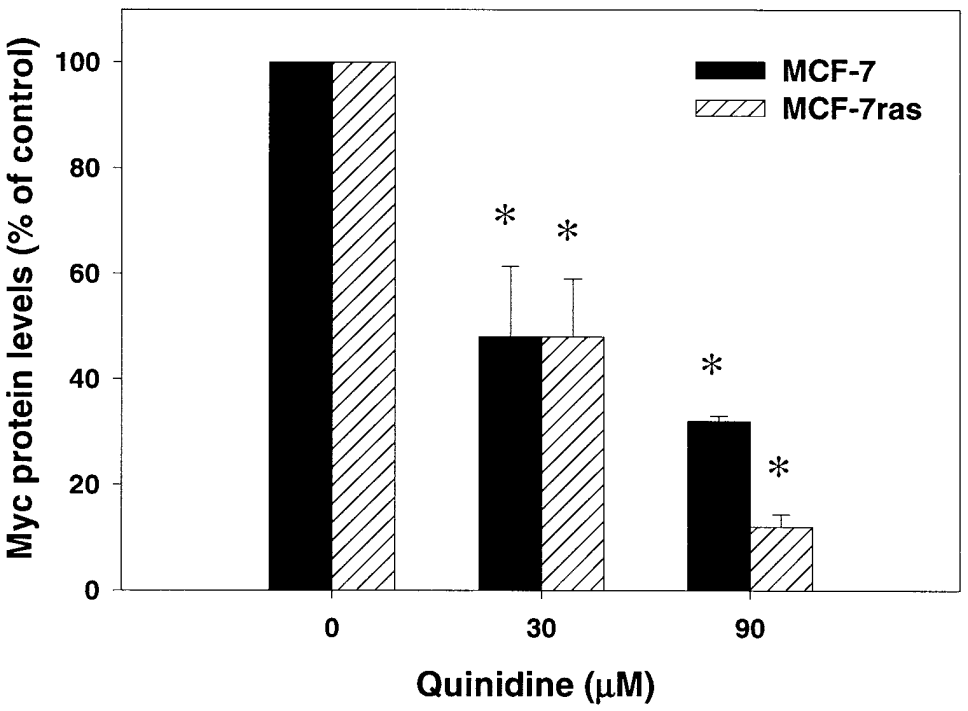


Figure 5B



Melkoumian, Z.K.

Figure 6A



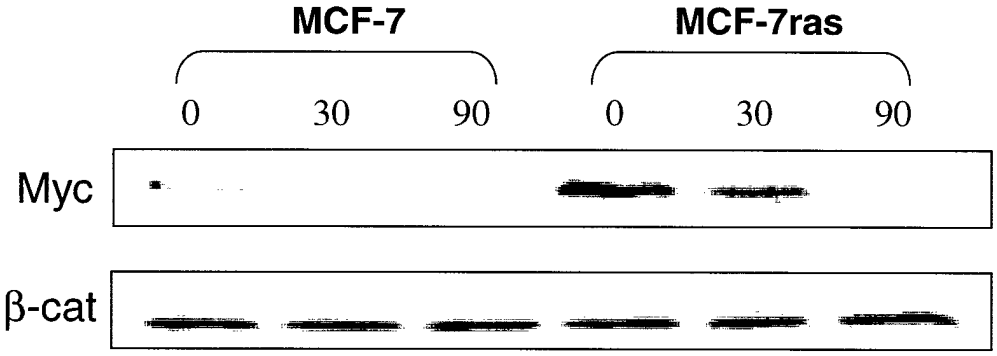
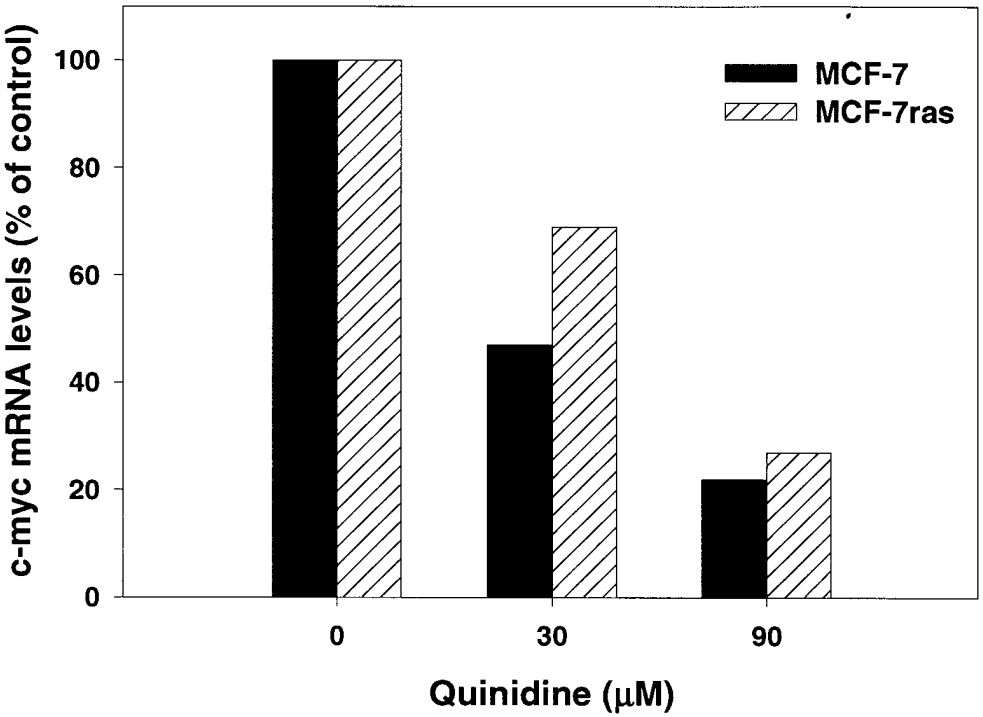
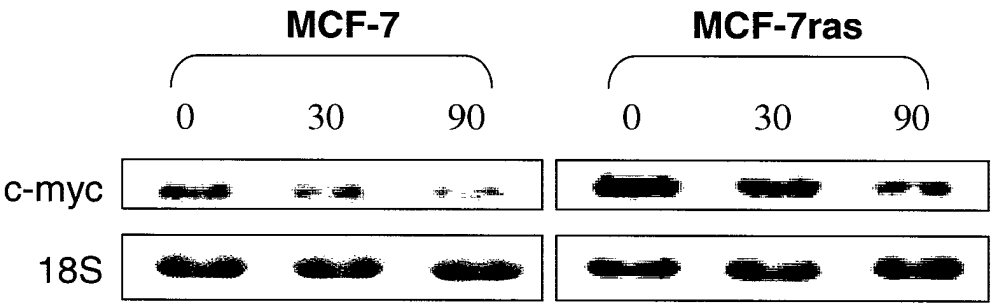


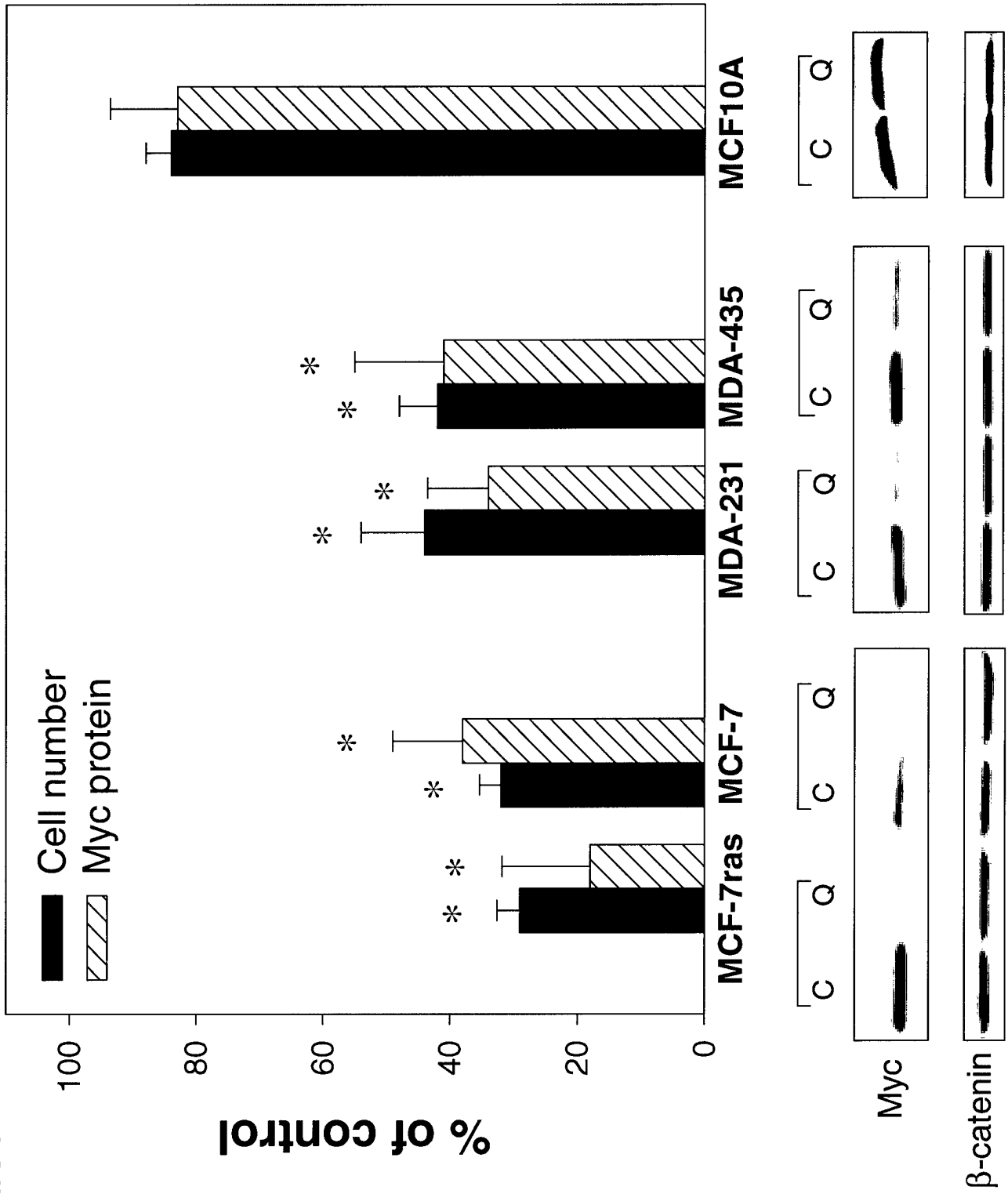
Figure 6B





Melkoumian, Z.K

Figure 7


Cell number
Myc protein
% of control
100
80
60
40
20
0
MCF-7ras
MCF-7
MDA-231
MDA-435
MCF10A
C
Q
Myc
β-catenin

Melkoumian, Z.K.

Figure 8

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Rapid Induction of Histone Hyperacetylation and Cellular Differentiation in Human Breast Tumor Cell Lines following Degradation of Histone Deacetylase-1*

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Quinidine inhibits proliferation and promotes cellular differentiation in human breast tumor epithelial cells. Previously we showed quinidine arrested MCF-7 cells in G_1 phase of the cell cycle and led to a G_1 to G_0 transition followed by apoptotic cell death. The present experiments demonstrated that MCF-7, MCF-7*ras*, T47D, MDA-MB-231, and MDA-MB-435 cells transiently differentiate before undergoing apoptosis in response to quinidine. The cells accumulated lipid droplets, and the cytokeratin 18 cytoskeleton was reorganized. Hyperacetylated histone H4 appeared within 2 h of the addition of quinidine to the medium, and levels were maximal by 24 h. Quinidine-treated MCF-7 cells showed elevated $p21^{WAF1}$, hypophosphorylation and suppression of retinoblastoma protein, and down-regulation of cyclin D1, similar to the cell cycle response observed with cells induced to differentiate by histone deacetylase inhibitors, trichostatin A, and trapoxin. Quinidine did not show evidence for direct inhibition of histone deacetylase enzymatic activity *in vitro*. HDAC1 was undetectable in MCF-7 cells 30 min after addition of quinidine to the growth medium. The proteasome inhibitors MG-132 and lactacystin completely protected HDAC1 from the action of quinidine. We conclude that quinidine is a breast tumor cell differentiating agent that causes the loss of HDAC1 via a proteasomal sensitive mechanism.

Histone deacetylase (HDAC)[1] proteins comprise a family of related proteins that act in conjunction with histone acetyltransferase proteins to modulate chromatin structure and transcriptional activity via changes in the acetylation status of histones. Histones H3 and H4 are the principal histone targets of HDAC enzymatic activity, and these histones undergo acetylation at lysine residues at multiple sites within the histone tails extending from the histone octamer of the nucleosome core. The association of HDAC proteins with mSin3, N-CoR, or SMRT and other transcriptional repressors has led to the hypothesis that HDAC proteins function as transcriptional corepressors (reviewed in Ref. 1). The spectrum of genes that show alterations in gene transcription rates in response to decreased HDAC activity is quite restricted (2). Yet, small molecule inhibitors of the enzyme histone deacetylase (HDAC) such as trichostatin A (TSA), superoylanilide hydroxamic acid (SAHA), trapoxin, and phenyl butyrate cause major alterations in cellular activity including the induction of cellular differentiation and apoptosis (3–5). Trichostatin A, SAHA, and trapoxin stimulate histone acetylation by acting as direct inhibitors of HDAC enzyme activity (6). Trichostatin A, SAHA, and trapoxin possess lysine-like side chains and act as chemical analogs of lysine substrates. Molecular models based upon the x-ray crystal structure of an HDAC-like protein indicate that trichostatin A and SAHA can bind within the active site of the HDAC enzyme and interact with a zinc metal ion within the catalytic pocket that is critical for enzymatic activity (7). Trapoxin is an irreversible HDAC enzyme inhibitor (8).

Much remains to be learned about the biochemical events subsequent to HDAC inhibition that lead to cell cycle arrest, cellular differentiation, and apoptosis. However, a spectrum of biological responses characteristic of HDAC inhibitors has emerged, including cell cycle arrest in G_1, elevated $p21^{WAF1}$ expression, hypophosphorylation of retinoblastoma protein (pRb), hyperacetylation of histones, particularly H3 and H4, and apoptosis. Histone hyperacetylation is directly linked to the activation of p21 transcription and is p53-independent (5). This observation provides an important link between HDAC inhibition and cell cycle arrest because $p21^{WAF1}$ plays a critical role in causing G_1 cell cycle arrest via inhibition of the G_1 cyclin-dependent kinase family (9). Overexpression of $p21^{WAF1}$ has also been associated with apoptosis, but the mechanism of $p21^{WAF1}$ induction of apoptosis requires further investigation (10).

Cancer therapy that targets the activity of genes or gene products controlling cell cycle progression, differentiation, and apoptosis is a promising new strategy. Because HDAC inhibitors regulate the cell cycle and cause both cellular differentiation and apoptosis, they comprise an interesting group of compounds with potential for development into a new category of clinically significant anti-tumor agents. Single, key protein targets for "gene-regulatory chemotherapy" are difficult to identify due to the existence of parallel, functionally overlap-

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[1] The abbreviations used are: HDAC, histone deacetylase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; PBS, phosphate-buffered saline; pRb, retinoblastoma protein; SAHA, superoylanilide hydroxamic acid; TSA, trichostatin A; HRP, horseradish peroxidase; HMEC, human mammary epithelial cells; ER, estrogen receptor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; CDK, cyclin-dependent kinase.

ping signaling cascades. For this reason, use of cancer therapies that target multiple intracellular signaling pathways, such as observed with the HDAC inhibitors, is an intriguing approach that addresses the problem of redundancy in growth signaling pathways. In this regard, the HDAC inhibitor phenyl butyrate was recently shown to have clinical anti-tumor activity (11).

Quinidine is a natural product therapeutic agent originally used as an anti-malarial and as an anti-arrhythmic agent. Previous studies with human breast tumor cell lines demonstrated that quinidine (90 μM) is an anti-proliferative agent as well. Quinidine arrested cells in early G_1 phase and induced apoptosis by 72–96 h in MCF-7 cells (12), but the biochemical basis for the anti-proliferative effect of quinidine was not well understood. To clarify the molecular mechanisms of the anti-proliferative activity of quinidine, we investigated the effects of quinidine on histone acetylation and cell cycle regulatory proteins. In this report, we show that quinidine causes hyperacetylation of histone H4, down-regulation of HDAC1 protein levels, and cellular differentiation in a panel of human breast tumor cell lines. We conclude that quinidine is a novel differentiating agent that stimulates histone hyperacetylation as a result of HDAC1 protein degradation.

MATERIALS AND METHODS

Cell Culture—Permanent cell lines derived from patients with breast carcinomas were used in these studies. MCF-7 cells, passage numbers 40–55, MCF-7*ras* (13), T47D, MDA-MB-231, and MDA-MD-435 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, Utah), 2 mM glutamine, and 40 μg/ml gentamicin. Experiments were performed in this medium supplemented with 5% FBS. The cells were maintained at 37 °C in a humidified atmosphere of 93% air, 7% CO_2. After 6 days, cells became about 70–80% confluent and were passaged at a 1:5 ratio (MCF-7) or at a 1:10 ratio (all others). Normal human mammary epithelial cells (HMEC) were obtained from Clonetics, San Diego, CA, and were grown according to directions of the suppliers. Cells were grown from frozen stocks and used for 1–3 passages. Quinidine, TSA, and all-*trans*-retinoic acid were purchased from Sigma. The cell-permeant proteasome inhibitors, MG-132 and lactacystin, were purchased from Calbiochem.

Growth Inhibition Assays—Growth inhibition by cell numbers was assayed by plating cells in 35-mm^2 dishes (1–1.5 $\times$ 10^5) containing DMEM, 5% FBS plus quinidine (90 μM). Viable cells were counted using a hemocytometer, and trypan blue (0.02%) exclusion was used as an indicator of viability. Cell growth was also monitored in a 96-well plate format using the One Solution Cell Proliferation Assay (Promega, Madison, WI), which is based upon metabolic bioreduction of a tetrazolium compound (Owen's reagent) to a colored formazan product that absorbs light at 490 nm. The plating density for the 96-well dishes (cells/well) was varied depending upon the relative growth rates of the cell lines as follows: HMEC (2000), MCF-7 (1000), MDA-MB-231 (500), T47D (1500), and MCF-7*ras* (500). The One Cell Proliferation Assay Reagent was added to each well and incubated for 2 h at 37 °C. Absorbance (490 nm) was read using a Molecular Devices PC340 (Sunnyvale, CA).

Microscopic Imaging—Cells were plated (1×10^5) on sterile coverslips in 35-mm^2 dishes and grown for 96 h in DMEM, 5% FBS supplemented with either 10 μM all-*trans*-retinoic acid (in 0.01% ethanol) or 90 μM quinidine (in H_20). Control cells were grown in medium containing a final concentration of 0.01% ethanol. The presence of ethanol had no effect upon lipid droplet accumulation compared with cells grown in DMEM, 5% FBS. Cells were fixed in 3.7% formaldehyde/PBS, rinsed in PBS (PBS: 140 mM NaCl, 2 mM KCl, 80 mM $Na_2HPO_4 \cdot 7H_2O$, 1.5 mM KH_2PO_4, pH 7.0), then treated briefly with 0.4% Triton X-100 in PBS. After rinsing three times in PBS, the cells on coverslips were incubated for 30 min at 37 °C with a primary antibody to cytokeratin 18 (1:1 dilution, provided by Dr. Guillaume van Eys, Maastricht University), rinsed, and incubated (30 min/37 °C) with Texas Red conjugated secondary antibody (goat anti-mouse IgG, Sigma). Alternatively, cells were incubated with fluorescein-phalloidin (1:200 dilution of a-5 μg/0.1 ml solution, Sigma) in the dark for 40 min at room temperature, rinsed, and incubated for 5 min (room temperature) with the fluorescent lipid stain, Nile Red (1:10,000 dilution of a 1 mg/ml acetone solution, Sigma) (14–15). All coverslips were rinsed in PBS and mounted with Fluoromount-G containing 2.5% *N*-propyl galate. Images were obtained using a Zeiss Axiovert 100 M confocal microscope ($\times$ 63 objective).

Immunoblotting—Cells were harvested from confluent T-75 flasks and subcultured (1×10^6) in 60-mm^2 dishes. On subcultivation, this confluent population of cells (85% in G_1) synchronously proceeded through the cell cycle. To prepare whole cell lysates, the cells were harvested at the times indicated by scraping into ice-cold buffer (50 mM Tris-HCl, 0.25 M NaCl, 0.1% (v/v) Triton X-100, 1 mM EDTA, 50 mM NaF, and 0.1 mM Na_3VO_4, pH 7.4). Protease inhibitors (protease inhibitor mixture, Roche Molecular Biochemicals) were added immediately. Cell lysates were centrifuged in an Eppendorf microcentrifuge (14,000 rpm, 5 min) at 4 °C, and the supernatants were used in immunoblotting experiments.

Histones were prepared from cells grown at a density of 1×10^7/T-162 flask. To harvest the cells, the flasks were placed on ice, and the growth medium was removed. Following a quick rinse with ice-cold PBS, cells were scraped into 1 ml of ice-cold lysis buffer (10 mM Tris-HCl, 50 mM sodium bisulfite, 1% Triton X-100 (v/v), 10 mM $MgCl_2$, 8.6% sucrose, pH 6.5) and nuclei released by Dounce homogenization. The nuclei were collected by centrifugation (3,000 rpm, 10 min, SS-34 rotor) and washed three times with the lysis buffer. Histones were extracted from the crude nuclear pellets using the procedure of Nakajima *et al.* (16). The pellets were resuspended in 0.1 ml of ice-cold sterile water using a vortex and concentrated H_2SO_4 to 0.4 N was added. The preparation was incubated at 4 °C for 1 h and then centrifuged (17,000 rpm, 10 min, Sorvall SS-34 rotor). The supernatant containing the extracted histones was mixed with 10 ml of acetone, and the precipitate was obtained after an overnight incubation at −20 °C, collected, and air-dried. The acid-soluble histone fraction was dissolved in 50 μl of H_2O and stored at −70 °C.

The protein concentration of the whole cell lysate supernatants or histone preparations was determined using the BCA protein assay (Pierce) and bovine serum albumin as a standard. Equal amounts of protein were loaded onto SDS-polyacrylamide gels. Molecular weights of the immunoreactive proteins were estimated based upon the relative migration with colored molecular weight protein markers (Amersham Pharmacia Biotech). Proteins were transferred to polyvinylidene difluoride membranes (NOVEX, San Diego, CA) and blocked at 4 °C using 5% nonfat milk blocking buffer (1 M glycine, 1% albumin (chicken egg), 5% non-fat dry milk, and 5% FBS) overnight. The membranes were incubated with primary antibodies for 3 h at room temperature. The antibody sources were as follows: mouse monoclonal anti-p27 (F-8, SC-1641), rabbit polyclonal anti-CDK4 (C-22), goat polyclonal anti-HDAC1 (N-19, SC-6299), all from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-pRb (14001A) from PharMingen (San Diego, CA); mouse monoclonal anti-cyclin D1 (NCL-cyclin D1, 113105) from Novocastra (Burlingame, CA); mouse monoclonal anti-p16 (Ap-1), p21 (WAF1,Ap-1), p53 (Ap-6) from Calbiochem; and anti-acetylated histone H4 antibody (rabbit polyclonal, Upstate Biotechnology Inc.). The primary antibodies were diluted at 1:500 in Western washing solution (0.1% non-fat dry milk, 0.1% albumin (chicken egg), 1% FBS, 0.2% (v/v) Tween 20, in PBS, pH 7.3). The antigen-antibody complexes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat IgG-HRP (SC-2020), rabbit IgG-HRP (SC-2004), or mouse IgG-HRP (SC-2005) from Santa Cruz Biotechnology) at a final dilution of 1:3000 in Western washing solution. After washing three times with Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.05% (v/v) Tween 20), antibody binding was visualized using enhanced chemiluminescence (SuperSignal West Pico, Pierce) and autoradiography.

In Vitro HDAC Activity Assay—Quinidine HCl was added to a chicken erythrocyte cellular extract, which contained HDAC activity, at concentrations of 90 μM (18). HDAC assays were performed as described in Hendzel *et al.* (17). Briefly, the cellular extract was incubated with 500 μg of acid-soluble histones isolated from [^{3}H]acetate-labeled chicken erythrocytes for 60 min at 37 °C. Reactions were terminated by addition of acetic acid/HCl to a final concentration of 0.12/0.72 N. Released [^{3}H]acetate was extracted using ethyl acetate and quantified by scintillation counting. Samples were assayed three times, and the non-enzymatic release of label was subtracted to obtain the reported values.

RESULTS

Hyperacetylation of Histone H4—Antibodies that recognize acetylated forms of histone H4 have been used as a probe for agents that cause histone hyperacetylation (19). In Western blot experiments, we compared the ability of quinidine to cause

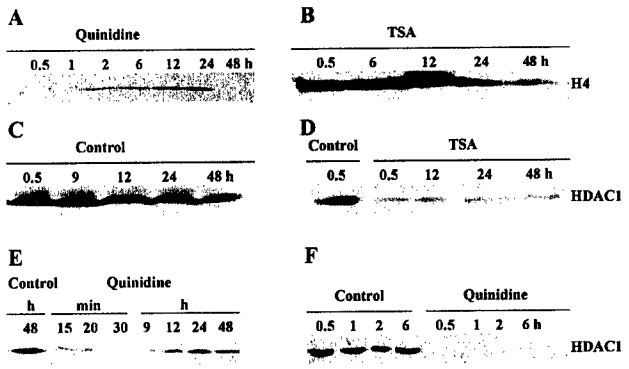

FIG. 1. **Histone hyperacetylation in MCF-7 cells.** *A,* histones were extracted from cells grown in the presence of 90 μM quinidine for 0.5, 1, 2, 6, 12, 24, or 48 h; histones (20 μg/lane) were electrophoresed in 15% polyacrylamide gels containing 1% SDS and assayed for the presence of acetylated H4 by immunoblotting. *B,* histones were extracted from cells grown in the presence of 300 nM TSA for 0.5, 6, 12, 24, or 48 h; 20 μg of histone/lane were electrophoresed and analyzed for acetylated histone H4 by immunoblotting. *C* and *D,* HDAC1 protein in whole cell lysates was prepared from control MCF-7 cells at 0.5, 9, 12, 24, or 48 h (*C*) or cells treated with 300 nM TSA 0.5, 12, 24, or 48 h (*D*); 50 μg of protein/lane were electrophoresed in 12% polyacrylamide gels containing 1% SDS and assayed for HDAC1 protein by immunoblotting. *E* and *F,* HDAC1 protein in whole cell extracts from cells grown in the presence of 90 μM quinidine for 15, 20, and 30 min or 9, 12, 24, or 48 h (*E*) or 0.5, 1, 2, or 6 h (*F*); extracts were electrophoresed (50 μg protein/lane) and assayed for HDAC1 protein by immunoblotting.

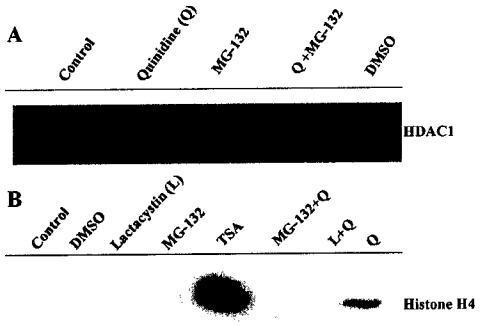

FIG. 2. **Protection of HDAC1 protein by proteasome inhibitors.** *A,* MCF-7 cells were released from confluency and subcultured in normal growth medium supplemented with 30 μM MG-132 in 0.1% Me_2SO (*DMSO*), 90 μM quinidine plus 0.1% Me_2SO or 0.1% Me_2SO alone as indicated. Cells were harvested after 30 min, and Western blot analysis of HDAC1 protein was performed as detailed in methods. *B,* MCF-7 cells were cultured as described above or with lactacystin (3 μM in 0.1% Me_2SO) except were harvested after 24 h and analyzed for the presence of immunoreactive acetylated histone H4.

hyperacetylation of H4 in MCF-7 cells with that of TSA, an established HDAC inhibitor, known to inhibit proliferation in MCF-7 cells (20). H4 acetylation in response to TSA was rapid (within 0.5 h) (Fig. 1*B*) and reached a maximum around 12 h. Some level of H4 acetylation persisted in the TSA-treated cells for 48 h. In cells treated with quinidine (Fig. 1*A*), detectable H4 acetylation was slightly delayed and could be seen at 2 h but not 1 h of treatment. H4 acetylation was maximal between 12 and 24 h but then sharply fell to an undetectable level at 48 h. Hyperacetylation of H4 was a transient response to both agents. Acetylated H4 is present in MCF-7 cells but under the conditions of Western blotting and immunochemical staining was not detected in control cells (data not shown).

HDAC1 is expressed in MCF-7 cells, and this enzyme contributes to the control of histone deacetylation rates (18). Quinidine caused the rapid disappearance of HDAC1 from MCF-7 cells. HDAC1 protein levels in quinidine-treated cells were reduced after 15–20 min compared with control cells, and HDAC1 protein was undetectable between 30 min and 6 h (Fig. 1, *E* and *F*). Partial restoration of HDAC1 protein occurred beginning at 9 h of treatment, but even after 48 h, HDAC1 levels in quinidine-treated cells were still less than control cells. Levels of HDAC1 protein in control MCF-7 cells were relatively constant during this time (Fig. 1*C*). TSA-treated MCF-7 cells also showed reduced levels of HDAC1 protein as early as 30 min after drug addition, and the reduced HDAC1 protein level was maintained through 48 h (Fig. 1*D*). The data indicate that loss of HDAC1 protein might contribute to the H4 acetylation response to both TSA and quinidine. However, HDAC1 protein levels were never reduced by TSA below the level of detection as was observed with quinidine. In light of the more extensive H4 acetylation response to TSA than quinidine, we conclude that the direct inhibition of HDAC1 catalytic activity by TSA remains an important component of the H4 acetylation response *in vivo*. In addition, the time course of the HDAC1 response to quinidine and TSA differ. In response to quinidine, there is initially a more marked decrease in HDAC1 protein levels but a more rapid recovery. TSA treatment caused a sustained reduction in HDAC1 protein levels through 48 h.

To determine if the rapid loss of HDAC protein in the presence of quinidine were mediated through the 26 S proteasome pathway, MCF-7 cells were treated simultaneously for 30 min with quinidine and MG-132 (30 μM), an inhibitor of the 26 S proteasome. Cells treated with 90 μM quinidine showed a complete loss of HDAC1 protein, which was prevented when MG-132 and quinidine were added simultaneously (Fig. 2*A*). Treatment with the solvent, Me_2SO, or MG-132 in solvent (0.1%) caused a modest reduction in the level of HDAC1 protein. These reductions in HDAC1 did not elicit a detectable stimulation of H4 acetylation, and we suggest that other HDAC enzymes present in MCF-7 cells, insensitive to Me_2SO, could compensate for the lost HDAC1 in the maintenance of deacetylated histone H4. This action of quinidine on HDAC1 protein was not reflected in a general decrease in cellular protein content (12), nor were all cell cycle regulatory proteins down-regulated in MCF-7 cells in the presence of quinidine (*e.g.* $p21^{WAF1}$ and p53 protein, Fig. 3). Additional studies are required to define the spectrum of proteins affected by quinidine in a proteasome-sensitive manner. Quinidine (90 or 250 μM) did not inhibit the activity of the isolated chicken erythrocyte HDAC1 enzyme *in vitro* (data not shown) suggesting that quinidine caused histone hyperacetylation by eliciting a rapid and transient loss of HDAC1 protein without a direct inhibition of the HDAC enzyme. The suppression of HDAC protein levels in MCF-7 cells was accompanied by a decrease in HDAC enzyme activity in the cell extracts. Histone acetylation and depressed HDAC1 protein levels persisted for approximately 48 h in the presence of quinidine. When MCF-7 cells were exposed to quinidine for 24 h in the presence of either MG-132 or lactacystin, there was no detectable H4 acetylation (Fig. 2*B*). These results support the idea that quinidine-induced loss of HDAC1 protein is involved in the H4 acetylation response via a proteasomal sensitive pathway.

G_1 Phase Cell Cycle Regulatory Profile in MCF-7 Cells—G_1 cell cycle arrest is characteristic of HDAC inhibitors, and reports of alterations in several cell cycle proteins in cells exposed to HDAC inhibitors, particularly the elevation of the $p21^{WAF1}$ protein, are numerous (21–23). It was of interest to determine whether $p21^{WAF1}$ and other key cell cycle regulatory proteins such as the retinoblastoma protein (pRb) and the G_1 phase cyclin-dependent kinase activator, cyclin D1, were targets of quinidine action in MCF-7 cells. Western blotting analysis showed that by 12 h the levels of $p21^{WAF1}$ were increased in response to quinidine treatment approximately 11-fold, and this elevated level of protein expression persisted through 48 h.

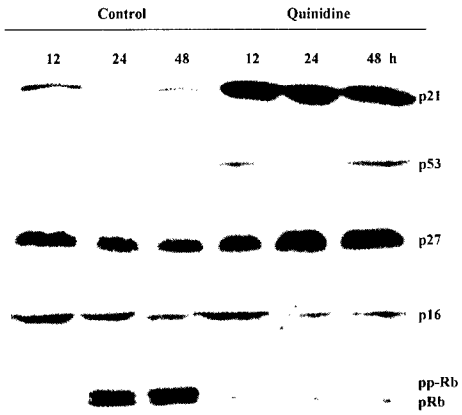

FIG. 3. **G_1 cell cycle proteins in MCF-7 cells.** Cells released from confluency were plated into control medium or medium containing 90 μM quinidine. Whole cell lysates were prepared 12, 24, or 48 h after plating and assayed by immunoblotting for the cyclin-dependent kinase inhibitors, $p21^{WAF1}$ (n = 3), p27 (n = 1), p16 (n = 3), and p53 (n = 3) after electrophoresis of 50 μg of protein/lane through 12% SDS-polyacrylamide gels. pRb protein was immunoprecipitated from 500 μg of whole cell lysate protein using an antibody that recognizes phosphorylated and non-phosphorylated pRb (56). This entire immunoprecipitate was electrophoresed in a 7.5% SDS-polyacrylamide gel and immunoblotted using this same antibody. Results shown are typical of two independent analyses.

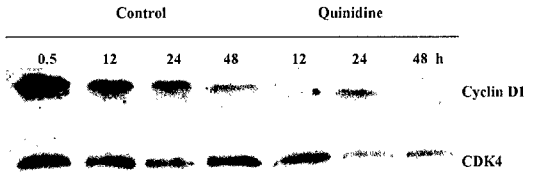

FIG. 4. **Cyclin D-CDK4 in MCF-7 cells.** Confluent MCF-7 cells were subcultured in control medium or medium containing 90 μM quinidine. Whole cell lysates were prepared 0.5, 12, 24, and 48 h after subculture. Equal protein aliquots (50 μg/lane) were electrophoresed in 12% SDS-polyacrylamide gels and assayed for cyclin D1 and CDK protein levels by immunoblotting. Results shown are representative of three independent experiments.

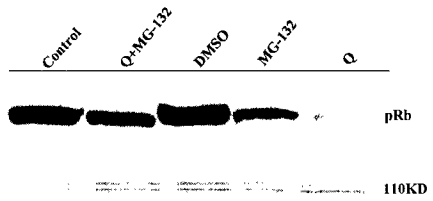

FIG. 5. **Proteasome inhibitor modulates retinoblastoma protein levels.** Confluent MCF-7 cells were subcultured in the presence of 90 μM quinidine, 30 μM MG-132, or quinidine + MG-132 for 24 h, then harvested, and whole cell extracts (100 μg/lane) were analyzed for pRb. A Coomassie Blue-stained protein is shown as the loading control.

A small, less than 2-fold increase in p27 levels was observed in cells exposed to quinidine for 24–48 h, whereas levels of p16 were unchanged (Fig. 3). Quinidine treatment decreased cyclin D1 and CDK4 protein levels after 12 h of treatment (Fig. 4), indicating that the cyclin-dependent kinase inhibitor, $p21^{WAF}$, as well as an important G_1 phase target of $p21^{WAF1}$, the cyclin D1-CDK4 complex, are early targets of quinidine in MCF-7 cells. This profile of activity is consistent with the observed cell cycle arrest of quinidine-treated MCF-7 cells in mid-G_1 phase (12).

In MCF-7 cell extracts probed using anti-pRb antibodies, two separate but closely migrating bands were distinguishable. The upper band contained more highly phosphorylated pRb, and the lower band contained unphosphorylated or hypophosphorylated pRb. Control cells showed a faint pRb signal at 12 h, typical of cells in early G_1 phase, and increased expression of both phosphorylated and unphosphorylated pRb at 24 and 48 h. Quinidine-treated MCF-7 cells had no detectable hyperphosphorylated pRb at any time point examined, and total levels of pRb protein failed to increase with progression through G_1 phase as seen in the control, proliferating cells (Fig. 3). The decrease in pRb phosphorylation level was predictable based on the increase in $p21^{WAF1}$ and decreased levels of both cyclin D1 and CDK4 (Fig. 4). In addition, Nakanishi *et al.* (24) showed that $p21^{WAF1}$ can bind pRb protein and block its phosphorylation. However, the actions of quinidine upon $p21^{WAF1}$ and cyclin D-CDK4 activity do not explain why the levels of total pRb protein were so low. Reductions in the cellular content of phosphorylated pRb protein in MCF-7 cells by quinidine is an important additional level of cell cycle control that effectively attenuates progression of cells out of G_1 phase and has been reported in other tumor cell lines in response to HDAC inhibition (22). In Fig. 5 we show data suggesting that the 26 S proteasome pathway regulates the total pRb content. MCF-7 cells incubated for 24 h in MG-132 or MG-132 plus quinidine had more total pRb than cells incubated with quinidine alone. Thus, quinidine promoted the loss of both HDAC1 and pRb, and inhibition of the 26 S proteasome pathway restored the levels of both of these proteins to that seen in the untreated cells. We have no direct evidence that quinidine promotes the proteasomal degradation of either protein. We hypothesize that quinidine may direct degradation of HDAC1 by the proteasome or, alternatively, quinidine might stimulate the proteasomal degradation of other regulatory factor(s) that act to maintain HDAC1 and pRb protein levels.

MCF-7 cells express wild-type p53 protein. Normal p53 is a short lived protein that is maintained at low levels, but in response to cell stress or DNA damage, p53 is stabilized and accumulates in the nucleus where it functions as a transcription factor inducing $p21^{WAF}$, G_1 cell cycle arrest, and apoptosis (25). Wild-type p53 down-regulates pRb levels in MCF-7 cells (26). Although Saito *et al.* (22) showed that p53 is not required for pRb down-regulation by HDAC inhibitors in all cell lines, quinidine-treated MCF-7 cells have elevated p53 levels (5–7-fold) (Fig. 3). Thus, p53 could contribute to the maintenance of the G_1 cell cycle arrest in MCF-7 by sustaining $p21^{WAF1}$ protein levels and suppressing pRb protein levels.

Growth Arrest and Cellular Differentiation in Human Breast Tumor Cell Lines—In contrast to MCF-7 cells, human breast tumor cell lines T47D, MDA-MB-231, and MDA-MB-435 express p53 proteins with distinct point mutations (27). To test for a requirement of p53, this panel of human breast tumor cell lines was exposed to quinidine, and the effects of quinidine on cell growth were compared (Fig. 6). The data shown are viable cell numbers/well, bioreductive metabolism/well, or both. In all four cell lines growth was suppressed in a concentration-dependent fashion between 10 and 90 μM quinidine, and maximal growth inhibition was observed at ~90 μM quinidine (data not shown). These data showed that growth suppression by quinidine is a p53-independent response. It is interesting that quinidine was not overtly cytotoxic in HMEC, a line of normal human mammary cells (28).

Evidence that quinidine elicited cellular differentiation in MCF-7 human breast tumor cells in conjunction with the inhibition of cell growth was obtained using maximally effective concentrations of quinidine or retinoic acid (data not shown). Antibodies directed against cytokeratin 18 (29) were used to probe the organization of the cytoskeleton (Fig. 7). In these studies, all-*trans*-retinoic acid (10 μM) was used to compare the differentiation response (30). Control MCF-7 cells showed expression of cytoplasmic cytokeratin 18 in a disorganized fash-

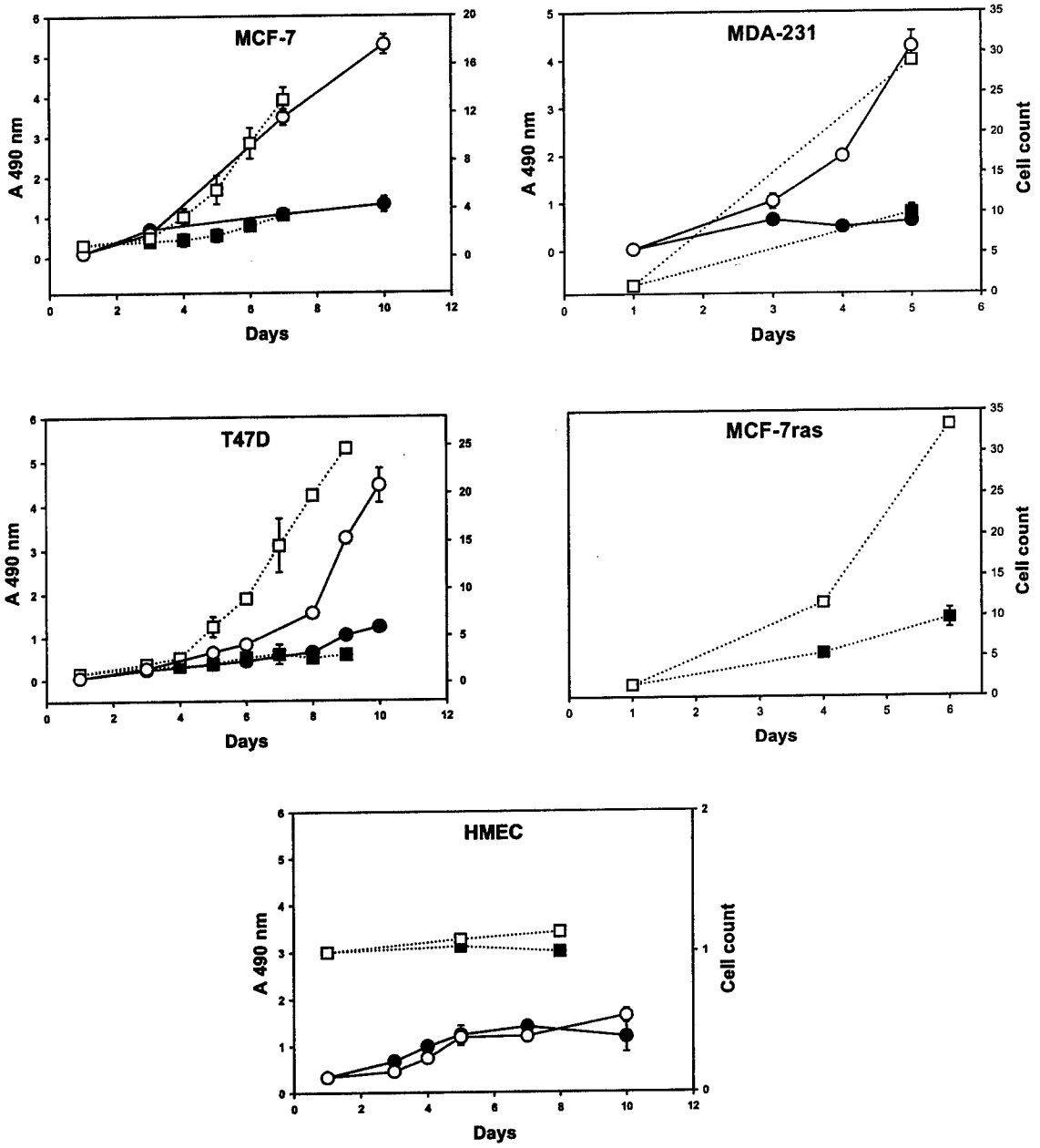

FIG. 6. **Growth of human breast cell lines in quinidine.** MCF-7, MDA-MB-231, T47D, MCF-7*ras* tumor cells, and normal HMEC were replica-plated in 96-well plates in control medium (*open symbols*) or medium containing 90 μM quinidine (*solid symbols*). Cell growth as monitored using the MTS assay is shown with solid lines. Results shown are the average of quadruplicates in one experiment. Quinidine had no effect on MTS metabolism in the MCF-7*ras* cell line (data not shown). Viable cell counts/dish of replica-plated MCF-7, MDA-MB-231, T47D, and MCF-7*ras* tumor cells and normal HMEC cells in 35-mm^2 dishes in control medium (*open symbols*) and medium containing 90 μM quinidine (*solid symbols*) is indicated by *dashed lines*. The cell number (growth curve data) represent the mean and S.E. of three independent experiments for the MCF-7, T47D, and MDA-231 cell lines performed in duplicate dishes (MCF-7 and T47D) or single dishes (MDA-231) for each experiment. MCF-7*ras* data are the mean (± range) of two experiments performed in duplicate dishes. HMEC data are from one experiment performed in single dishes.

ion. Cells that were treated for 96 h with retinoic acid showed an increase in the intensity of the cytokeratin 18 staining and relocalization of cytokeratin 18 throughout the nucleus as well as the cytoplasm. In contrast, cytokeratin 18 staining occurred in a highly organized pattern in MCF-7 cells treated with quinidine for 96 h and the cells adopted a shape and nuclear localization more typical of columnar epithelium.

Lipid droplets are found in the cytoplasm of normal mammary epithelium (31), and cytoplasmic lipid droplet accumulation occurs in a variety of differentiating cell systems. Induction of differentiation in human breast cancer cell lines by oncostatin M (32), the HER-2/neu kinase inhibitor, emodin (33), overexpression of c-e,rbB-2 (34), the vitamin D analog, 1-α-hydroxyvitamin D5 (35), the HDAC inhibitor, sodium butyrate (36), and retinoic acid (36) is accompanied by the accumulation of cytoplasmic lipid droplets. We utilized a fluorescent stain, Nile Red to monitor lipid droplet formation in mammary tumor cells in response to quinidine. The cells were counterstained with fluorescein-phalloidin that binds actin filaments to assay for changes in the actin cytoskeleton (Fig. 8). The distribution of actin in four human breast tumor cell lines, MCF-7, T-47D, MDA-MB-231, and MDA-MB-435 is seen clearly in the control cells. Three of these lines show strong nuclear staining of actin characteristic of transformed cells, whereas the fourth, MDA-MB-435, shows more cytoplasmic actin. In all cases except MDA-MB-435, the presence of quinidine did not significantly alter the actin cytoskeleton. Lipid droplet accumulation was weak or absent in the control cell

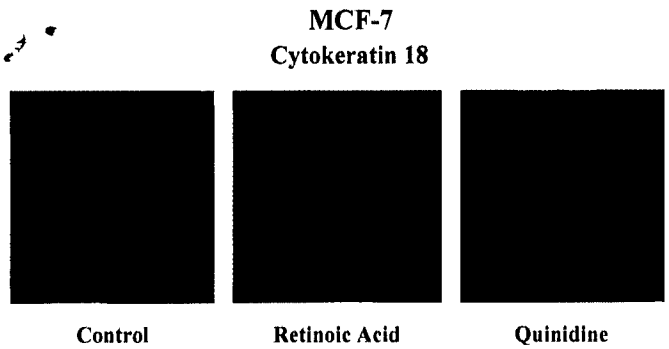

FIG. 7. **Cytokeratin 18 in MCF-7 cells.** Cells were replica-plated (2×10^5) on sterile coverslips in 35-mm^2 dishes in medium containing 0.01% ethanol (control), 10 μM retinoic acid, or 90 μM quinidine and grown for 96 h. Cytokeratin 18 detection using a Texas Red-tagged secondary antibody is shown using confocal microscopy. Data shown are typical fields representative of two independent experiments.

lines and increased by retinoic acid and quinidine. Lipid droplet accumulation was more marked in all four cell lines treated with quinidine than with retinoic acid. These data demonstrate that induction of a more differentiated phenotype is a general response of human mammary tumor cells to quinidine.

Hyperacetylation of Histone H4 in Mammary Tumor Cell Lines by Quinidine—To determine whether differentiation and histone acetylation were linked, we investigated the histone H4 acetylation status of quinidine-treated T47D, MDA-MB-231, and MCF-7_{ras} cells. MCF-7, MCF-7*ras*, T47D, and MDA-MB-231 cells were incubated for 24 h in the presence or absence of quinidine, and then histones were extracted for immunoblotting. Fig. 9 shows that histone H4 was hyperacetylated in all cell lines treated with quinidine. Control cells contained no hyperacetylated histone H4.

DISCUSSION

Quinidine-induced histone H4 hyperacetylation in MCF-7 human breast carcinoma cells can be attributed to the rapid elimination of HDAC1 protein, a response that was blocked by MG-132 and lactacystin, two inhibitors of proteasome-mediated proteolysis. HDAC1 protein was undetectable within 30 min after the addition of quinidine to the medium of MCF-7 cells, and hyperacetylated histone H4 appeared between 1 and 2 h. Levels of HDAC1 protein were completely suppressed between 0.5 and 6 h, and during this time H4 acetylation levels increased. H4 acetylation was maintained at 12 and 24 h, despite the partial restoration of HDAC1 protein at these same time points. These data indicate that quinidine-induced reductions in HDAC1 protein levels are unlikely to explain fully the regulation of H4 acetylation state in MCF-7 cells by quinidine. Additional HDAC enzymes or effects upon histone acetylation rates could possibly play a role as well.

An earlier study showed that over this initial 48-h period, 80% of the MCF-7 cell population had shifted into G_0, a quiescent state marked by the absence of Ki67 antigen immunoreactivity (12). Cellular differentiation manifested as the accumulation of lipid droplets, and a reorganization of the cytokeratin 18 cytoskeleton was evident after this initial 48-h period. Quinidine exhibited all the responses typical of known HDAC inhibitory drugs, with the exception that quinidine had no direct inhibitory effect upon HDAC1 enzymatic activity. We conclude from the current studies that quinidine is a novel differentiating agent that causes histone hyperacetylation, in part, by physical elimination of HDAC1 protein rather than the inhibition of HDAC enzymatic activity.

Histone H4 hyperacetylation and induction of cellular differentiation by quinidine were seen in a panel of human breast tumor cell lines that were selected for study on the basis of their diversity of genetic backgrounds. The differentiation response to quinidine was independent of the estrogen receptor (ER) status. Cell lines representative of ER-positive and ER-negative human breast carcinoma cells were induced to differentiate in the presence of quinidine. The ER status of the estrogen receptor positive cell lines is MCF-7 (ER-α and ER-β), T47D (ER-α and ER-β), and MDA-MB-231 (ER-β). MDA-MB-435 cells expressed very low levels of ER-β and no ER-α (37, 38). MCF-7 and T47D cells display an epithelial morphology and show similarities with mammary ductal and luminal epithelial cells, respectively (30, 39). MDA-MB-231 cells exhibit an elongated cellular morphology that is also typical of MDA-MB-435 cells. Our results demonstrate that quinidine is a differentiation agent in both types of mammary tumor cells.

HDAC inhibitors reverse the transformed phenotype of NIH3T3*ras* cells, and this property has been used successfully as a screening assay for the identification of new HDAC inhibitors (40, 41). Quinidine elicited a more differentiated phenotype in MCF-7*ras* cells, an MCF-7 cell derivative produced by stable transformation with v-Ha-*ras,* thus demonstrating that quinidine, like other HDAC inhibitors, can reverse an Ha-*ras*-induced phenotype.

Quinidine induced differentiation independently of wild-type p53. The ability of quinidine to cause differentiation of p53 mutant cell lines is consistent for a role of histone hyperacetylation in the response. HDAC inhibitors typically induce a p53-independent activation of $p21^{WAF1}$ gene expression (5, 22). Growing MCF-7 and T47D cells express $p21^{WAF1}$ protein in moderate to low levels (42), and quinidine raised $p21^{WAF1}$ protein levels in MCF-7 cells approximately 11-fold within 12 h. Although $p21^{WAF1}$ was reported to be low to undetectable in MDA-MB-231, $p21^{WAF1}$ was detected in Western analyses of both MDA-MB-231 and T47D cells in a p53-independent fashion in response to serum deprivation, adriamycin, etoposide (42, 43), and quinidine (data not shown). These data support the idea that the $p21^{WAF1}$ gene is present but inactive in growing MDA-MB-231 cells. Since histone hyperacetylation of the $p21^{WAF1}$ gene occurs in response to HDAC inhibitors, it might be involved in the pathway of p53-independent activation of $p21^{WAF1}$ gene expression (5).

The processes of cellular differentiation and cell cycle progression are interdependent. G_1 arrest is a necessary but insufficient condition for differentiation in numerous cell types including leukemic cells, keratinocytes, colonic epithelium, and muscle cells. In all of these cells, induction of $p21^{WAF1}$ protein and G_1 cell cycle arrest occurred prior to differentiation (44–50) and was generally independent of p53. We hypothesize that the differentiated state can be viewed as a cellular response to G_1 arrest, requiring a change in gene expression profile and suppression of cell death pathways. The response of MCF-7 breast tumor cells to quinidine is consistent with this model.

To begin to understand how quinidine might elicit G_1 arrest of MCF-7 cells, we have focused on the action of quinidine as a potassium channel blocking agent. Quinidine enters cells and inhibits cardiac potassium channels by binding to the intracellular face of the ion pore (51). Although the location of the quinidine-binding site on the ATP-sensitive potassium channel is unknown, quinidine is freely permeable across membranes and inhibits the ATP-sensitive potassium channels whether it is applied to the external or internal surface of a lipid membrane bilayer (52).

In the presence of quinidine, MCF-7 cells accumulate at a position 12 h into G_1 phase (12). This position, defined by cell cycle arrest and release experiments, precedes the lovastatin arrest point by 5–6 h and is clearly distinct from the restriction

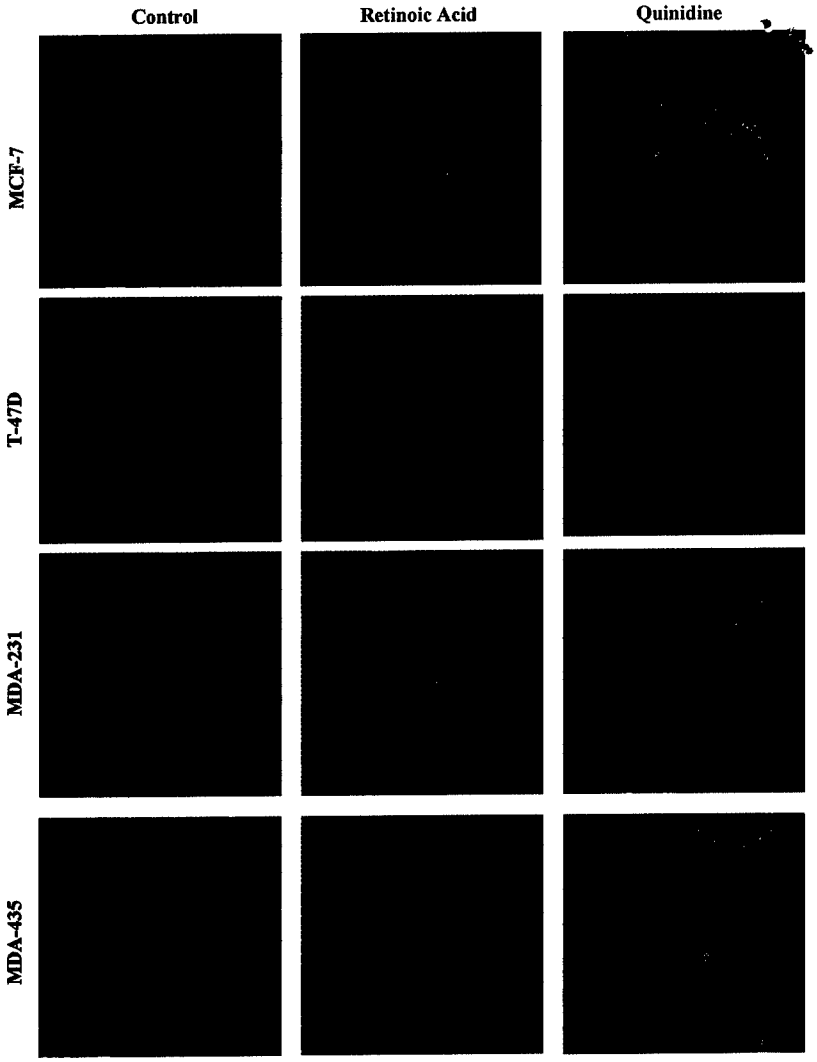

FIG. 8. **Lipid accumulation as an index of cellular differentiation in human breast tumor cell lines.** MCF-7, T47D, MDA-MB-231, and MDA-MB-435 cells were replica-plated ($0.8–3 \times 10^5$) on sterile coverslips in 35-mm^2 dishes in medium containing 0.01% ethanol (control), 10 μM retinoic acid (0.01% ethanol), or 90 μM quinidine (in water). Cells were fixed, permeabilized, and then incubated sequentially with fluorescein-phalloidin to identify actin filaments and Nile Red to identify lipid droplets after 96 h. Images were obtained by confocal microscopy. The results are typical of three experiments conducted in each cell line.

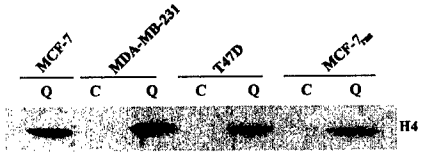

FIG. 9. **Histone H4 hyperacetylation in human breast tumor cell lines.** MCF-7, MCF-7*ras*, T47D, MDA-MB-231, tumor cells were replica-plated (1×10^7/T-162 flask) in control medium (*C*) or medium containing 90 μM quinidine (*Q*). Histones were extracted from the cells after 24 h, and 20 μg/lane of histone proteins were electrophoresed in 15% SDS-polyacrylamide gels. Immunoblotting was performed to detect acetylated histone H4.

point described by Pardee (53) near the G_1/S transition. The present work showed that quinidine treatment caused elevated levels of p53 and p21^{WAF1} protein by 12 h (Fig. 3), the point within G_1 where MCF-7 cells arrest in response to quinidine (12). When p53 and p21^{WAF1} proteins were assayed before 12 h, p53 was undetectable, and p21^{WAF} was first detected after 8 h of quinidine treatment (data not shown), suggesting a p53-independent induction of p21^{WAF1} occurred prior to arrest in G_1. CDK4 and cyclin D1 protein levels were also reduced, as was CDK4 activity as demonstrated by the abundance of hypophosphorylated pRb protein. Based upon our observations in MCF-7 cells, we conclude that p21^{WAF1} protein levels become elevated prior to the G_1 arrest in response to quinidine and could initiate the G_1 arrest. Hypophosphorylated pRb protein is prominent in quinidine-treated MCF-7 cells, and this could act to sustain the G_1 state by preventing the transition into S phase. The G_1 arrest induced by quinidine in MCF-7 cells was correlated with the blockade of ATP-sensitive potassium channels in MCF-7 cells (12, 54, 55). Direct evidence for the involvement of potassium ions in the G_1 arrest was provided using valinomycin, a potassium-selective ionophore to stimulate a G_1-S phase transition in the presence of quinidine (12).

In summary, quinidine, a drug that is used therapeutically in the treatment of malarial infections and cardiac arrhythmia, was shown to be useful as an inducer of cellular differentiation in human breast tumor epithelial cells. Quinidine caused histone H4 hyperacetylation and cellular differentiation in human breast tumor cells following the rapid loss of HDAC1 involving a proteasome-dependent pathway. Additional experiments are needed to determine how the action of quinidine upon ATP-sensitive potassium channels initiates the molecular events underlying the differentiation response.

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